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INTRODUCTION

An important problem for many biological scientists is that of determining the mechanisms whereby organisms record and store information relative to their life experiences. For a number of years psychologists and others have suggested that neurological changes occur during learning, these changes representing memory. Also there has been the expectation that some molecular modification would underlie these neurological events. Thus the modified molecule would be the mechanism for symbolizing the experiential events. Any one of a number of molecules might be involved or altered in the memory process.

Molecular biologists have made rapid strides in recent years. For structural genes the transfer of genetic information during protein synthesis which involves deoxyribonucleic acid (DNA), a number of ribonucleic acids (RNA), and amino acids has been well analyzed. Tentative RNA codes have been proposed for each amino acid. An air of excitement and enthusiasm seems to permeate researchers with the expectation that man is getting closer and closer to nature's basic secrets.

The developments in molecular biology have had a stimulating effect on the thinking and experimental research of a number of psychologists and other biological scientists and have led to hypotheses concerning the involvement of DNA and RNA (and other macromolecules) in learning and memory. This report contains papers presented during a scientific conference involving prominent molecular biologists, neurobiologists, and psychologists in which important developments concerning the role of nucleic acids and other macromolecules in complex behavior such as learning were discussed. The conference provided an opportunity for the participants and attendees to critically analyze research and ideas in this area and to suggest new ideas and research

efforts. The principal investigator wishes to thank Drs. Gilbert Tolhurst and Joseph Saunders of the Office of Naval Research for their interest in this stimulating and provocative conference.

A CELLULAR APPROACH

TO ADAPTIVE PROCESSES IN THE NERVOUS SYSTEM

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The phenomenon of complex psychological behavior is an evolutionary development which occurs only in organisms which possess a nervous system, composed of cells which are organized to regulate adaptive behavior. Thus, if this symposium is addressed toward understanding biochemical processes which underlie the phenomenon of adaptive behavior, it must of necessity deal both with problems of molecular organization within cells and with problems of cellular organization within the nervous system. Memory, learning, or any other aspect of complex psychological behavior cannot be explained solely on the basis of the properties of an individual class of macromolecules. For if one were to ask today, "What macromolecules show properties which can handle the requirements of complex behavior?" one would have to state, on the basis of current knowledge, that no individual type of macromolecule is capable of handling this task. It is only when these macromolecules are functioning in an integrated, cellular system that the phenomenon of complex behavior occurs. We have recently presented an extensive discussion of this problem (Dingman & Sporn, in press).

If one wishes to deal with the phenomenon of complex behavior, it is therefore essential to consider both the interactions that occur between metabolic components within brain cells and the interactions that occur between cellular components of the nervous system. In this physiological framework there appear to be two major problems, regarding the nature of cells of the nervous system, that need to be answered:

First: in what manner is the neuron (or a group of neurons and

associated glia) specialized to receive information from its cellular environment? This question leads both to an analysis of the specialized aspects of the neuronal membrane and to an analysis of transient changes that occur within cells of nervous tissue, as a result of functional stimulation.

Second: In what manner is the neuron (or a group of neurons with associated glia) specialized to store information coming from its cellular environment? Since a storage function without a "readout" mechanism is of no adaptive significance, one may ask the corollary question, "In what manner is a neuron (or a group of neurons with associated glia) specialized to make a permanent change in its own output, as a result of functional stimulation?" Does this permanent change involve the synthesis of qualitatively new molecules, or is it limited to changing the relative proportions and spatial distribution of molecules in the nervous system? Therefore we must find a mechanism, unknown at present, whereby the transient inputs of functional stimulation are able to cause a permanent change in output.

In this context, it is apparent that modern tools of molecular analysis will have much to contribute to our future understanding of complex behavior. But until we can organize molecular concepts into a physiological, cellular framework, we will lack a coherent picture of how molecular events result in what we observe as behavior. Unfortunately, our current knowledge of many aspects of the cellular physiology of neurons and glia is very limited. In the following discussion, I would like first to give a necessarily brief summary of what, in general, is known regarding the two major problems just mentioned, and then to discuss some tentative approaches to these problems that we have been exploring in our own laboratory.

We shall begin with the second, and more difficult, problem, the one of information storage and readout. How, in fact, does a neuron make a

permanent change in its own output, as a result of functional stimulation?
In terms of a modern approach to this problem, a hypothesis that was sug-
gested by Cajal (1910) over 50 years ago, and later modified by Hebb (1949),
Sholl (1956), Weiss (1961, P. 220), and others, is still a very timely and
important one. The hypothesis in its broadest sense states **first**: that the
neuron is a cell specialized for cytoplasmic growth during its entire func-
tional lifetime and **second**: that growth of new or larger or more effective
synaptic connections is an essential feature of memory storage and learning.
There is by now a wealth of experimental evidence to support the first part
of the hypothesis, that the neuron is a cell specialized for cytoplasmic
growth; we will summarize this evidence shortly. Moreover, modern biochem-
ical techniques are continually adding more data to this already impressive
evidence. However, we cannot as yet present such a clear-cut picture to
support the second part of the hypothesis, that is if we wish to establish
a mechanistic relationship between neuronal growth and storage of informa-
tion. The growth hypothesis would postulate that with functional activity
new or larger or more effective synaptic connections are formed, and that
these changed connections may be regarded as the actual memory traces. It
would be nice indeed if one could present a mass of data which support a
temporal or causal connection between growth in the nervous system and the
capacity for complex behavior. This cannot be done at present, but it is
an exciting possibility for the future.

However, let us summarize some of the data that tell us that cell growth
is an important phenomenon in brain. One may start with the classical
Golgi studies of the cerebral cortex (Conel, 1939, 1941, 1947), which show
a great proliferation of neuronal cytoplasm (in the form of new dendritic
and axonal connection) during the first few months of life. Modern electron
microscopic studies confirm the older Golgi studies (Voeller, Pappas, and

Purpura, 1963). The importance of adequate sensory stimulation for neural growth is well known (Mendelson and Irwin, 1962), and furthermore, it appears that the functional requirements of the periphery can determine the growth pattern of axonal termini (Edds, 1953). Growth and/or regeneration of axons and dendrites during adult life has been shown in many studies of axoplasmic flow in peripheral neurons (Droz and Leblond, 1962 and Ochs, Dalrymple, and Richards, 1962) and in recent work by Rose and his colleagues on regeneration in central neurons (Rose, Malis, Kruger, and Baker, 1960). Still further support for the growth model comes from recent cytochemical and biochemical studies on brain, in that it appears that the general capacity for synthesizing RNA (Hyden, 1960, P. 215 and Hay and Revel, 1963), proteins (Palay and Palade, 1955 and Waelsch and Lajtha, 1961), and lipids (Rossiter, 1957, P. 355 and Brady, 1960) is extremely high in brain. Generalized cell growth requires a generalized ability to synthesize a wide variety of molecules, and the more one looks at brain, the greater its general synthetic capacities appear to be. Yet it spite of its great synthetic capacities, there is no good evidence at present that brain is specialized to make qualitatively new molecules as a result of functional stimulation. In particular, there appear to be no biochemical data that memory storage is associated with the specific formation of a qualitatively new and specific DNA, RNA, or protein molecule, which may be regarded as an actual engram of memory. From a biochemical point of view, it is much simpler to think of functional stimulation as changing the relative proportions and spatial distributions of molecules in the nervous system than to postulate the production of a whole new class of molecules which serve as permanent memory traces.

A generalized growth mechanism linked to functional stimulation therefore becomes an extremely attractive model for storage of information. The

problem then becomes, "How can one test the proposition that transient changes of functional stimulation cause growth?" This problem takes us back to the first question we asked some time ago, namely, "What transient changes occur in neurons and glia, as a result of functional stimulation?" The extremely important problem of the role of the nerve membrane in excitation will be discussed shortly by Dr. Tobias, who has done a great deal of work in this field, and I would not presume to discuss this particular topic. However, it must be emphasized that important transient changes also occur with functional stimulation in the cytoplasm itself, as well as at the membrane. Many investigators have studied this problem, and one of the best systems thus far devised is the isolated sympathetic ganglion preparation of Larrabee and his collaborators (Larrabee, 1958; Horowicz and Larrabee, 1958; Horowicz and Larrabee, 1962). In an extensive series of experiments they have shown that functional activity of the ganglion definitely increases its energy requirements; thus, with activity there is a major increase in oxygen and glucose uptake and in lactate and carbon dioxide production.

Another important change that occurs in nervous tissue with functional activity is an increase in the free ammonia level. The elevation of the free ammonia level in stimulated peripheral nerve has been known for many years (Tashiro, 1922; Winterstein and Hirschberg, 1925; Gerard, 1932); more recent work has indicated that functional stimulation also causes an elevation of free ammonia or free glutamine in the brain itself (Richter and Dawson, 1948; Vrba, 1956; Tsukada, Takagaki, Sugimoto, and Hirano, 1958). Whether the increased level of free ammonia is the result of increased production by certain pathways or decreased utilization by other pathways still remains to be solved. In summary, functional activity of brain causes transient, but important, changes in carbon dioxide production and increases in the level of free ammonia in the tissue. With regard to these alterations

in both carbon dioxide and ammonia, recent neurochemical investigations with intact animals (Berl, Takagaki, Clarke, and Waelsch, 1962; Vrba, Gaitonde, and Richter, 1962; Sporn, Dingman, and Defalco, 1959) have emphasized the importance of the amino acids glutamate, glutamine, and aspartate for carbon dioxide and ammonia fixation. It has been shown that in the intact animal the cerebral pools of these three amino acids are in equilibrium with the cerebral pools of carbon dioxide and ammonia, as well as having important metabolic relationships with glucose and other amino acids. Thus, alterations in the free carbon dioxide and free ammonia pools will rapidly be reflected in the cerebral pools of glutamate, glutamine, and aspartate.

What implications do these findings have for cytoplasmic growth? What are the possible connections between carbon dioxide, ammonia, glutamate, glutamine, aspartate and the ability of a neuron to form new cytoplasm? Biochemically, it is well established that any cytoplasmic growth mechanism will require, among other things: 1) the synthesis of purines and pyrimidines, which are basic constituents of RNA, 2) the synthesis of proteins, and 3) the synthesis of lipids. We do not have complete information on all of these processes (particularly pyrimidine synthesis) in brain, but there is already enough data to suggest that fixation of carbon dioxide and ammonia during functional activity may be an important stimulus to cytoplasmic growth. With respect to carbon dioxide fixation, the following is known: carbon dioxide may be fixed in brain into precursors of RNA (Berl et al 1962; Henderson and LePage, 1959), proteins (Berl et al 1962), and the fatty acids of lipids (Brady, 1960; Waite and Wakil, 1962). Likewise ammonia is also fixed in brain into precursors of RNA (Berl et al, 1962; Henderson and LePage, 1959) and proteins (Berl et al, 1962). In most of these reactions, glutamate, glutamine, and aspartate play central roles, although the carbon dioxide fixation during fatty acid synthesis appears not to involve these

amino acids directly. In the stimulation of fatty acid synthesis, both carbon dioxide and citrate, an important metabolite of glucose, play key regulatory roles (Brady, 1960; Brady and Gurin, 1952; Vagelos, Alberts, and Martin, 1963). Inasmuch as the utilization of glucose and the concentration of citrate in the cell may be determined by the degree of functional activity, again we have a possible mechanism whereby functional activity may control growth.

It should be apparent, then, that this entire field is ready for some very intriguing experiments. We know that the production of carbon dioxide and the level of ammonia is increased in the brain with functional activity, and we also know that carbon dioxide and ammonia may be fixed into precursors for cellular growth. That, however, does not prove that there is actually net growth by this mechanism during functional activity. Experimentally, one still has to demonstrate that functional activity directly causes increased purine, pyrimidine, and RNA synthesis, increased protein synthesis, increased lipid synthesis, and new, larger, or more efficient synaptic connectivity.

Unfortunately, much of the experimental work directed toward this end, which has used radioisotopes (with and without radioautography) to measure rates of reactions in the intact animal, has failed to yield data that can be adequately interpreted. For until someone solves the troublesome problems of cellular compartmentation and measurement of true isotopic precursor concentrations within cells, any attempt to measure rates of synthetic reactions in the brain, using radioisotopes in a live animal, appears bound to fail, as has been elegantly demonstrated by both Reiner (1953) and Russell (1958). This is by no means a nihilistic approach to the problem, for there are certainly many other ways that the problem may be attacked. The devising and application of such alternative approaches

to measuring critical biochemical changes that occur during functional
activity represent an exciting and promising problem for the future.

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A CHEMICALLY SPECIFIED MOLECULAR

MECHANISM UNDERLYING EXCITATION

IN NERVE: A HYPOTHESIS

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Nerve Excitation

How an axon shifts from the resting to the excited state can be discussed in terms of three groups of phenomena. First, there is depolarization. Secondly, there is a complex of sub-molecular, molecular and macromolecular level events which are initiated by the depolarization. Thirdly, if the depolarization is sufficient the second class of events grows to a point where membrane resistance falls and the ion fluxes characteristic of the fully developed excited state (Hodgkin, 1951; Huxley, 1959) are permitted to occur. These three sets of changes overlap and interact in time, but can be discussed separately. The word "excitation" refers to the first and second sets.

Research emphasis for many years has been focused primarily on phases 1 and 3 above, and much is known about their important electrophysiological and electroionic components (Hodgkin, 1951; Huxley, 1959; Cole and Curtis, 1939), whereas relatively little work has been done on phase 2 which is now so greatly challenging. Nevertheless, enough information is at hand so that it should be fruitful clearly to state a limited but chemically specified working hypothesis about phase 2, to collate the relevant evidence, to

verbalize certain questions raised by the hypothesis, and to notice some of the experiments it suggests. Although it is pertinent for other excitable cells and for junctions, and although it is suggestive for recovery phenomena, the hypothesis will, for the sake of simplicity, be presented only in terms of axonal excitation.

Statement of Hypothesis

The early, catelectrotonic, outward, exciting current moves potassium from the axoplasm into the membrane phase. This intruding potassium displaces calcium from polar carboxyl and/or phosphate groups on membrane phosphatidylserine. As one consequence, water content increases in the membrane phase. The combined calcium displacement and increased hydration cause, and are paralleled by, loosening and spreading apart of membrane structural components, phospholipid being the most important. A change in geometry of the membrane protein increases this spatial deformation. The three events: (a) catelectrotonic injection of potassium into the membrane phase to displace calcium (ion exchange); (b) water moving into the membrane phase (hydrokinesis); (c) protein deformation of the membrane (proteokinesis), are responsible for the reversible change in membrane structure which expresses itself as a fall in membrane resistance permitting the ion fluxes of activity to supervene.

Separation of and Evidence for Propositions Explicit or Implicit in the Hypothesis

Proposition 1. Catelectrotonic, outward cation flow, not anelectrotonic inward cation flow, is exciting: (a) The evidence that excitation occurs where positive current flows out of the cell cathodally, and not where it flows inward anodally, is too extensively documented to require support here (Rushton, 1949; Hodgkin, 1937).

(b) The argument that it is an ion displacement current which excites

does require clarification, since it is often stated that ' . . . in the squid axon the three variables, sodium conductance, potassium conductance and inactivation, are governed solely by membrane potential. . . ." (Shanes, 1958). This can be misinterpreted, and is sometimes considered to mean that ion displacement current has been excluded as a causal factor in the excitation process. In fact, however, when the membrane potential is suddenly reduced (depolarization), an initial pulse of capacity current precedes the large currents carried chiefly by sodium and potassium moving down their own electrochemical gradients, and to account qualitatively for the propagation of an action potential the sequence of events at each point on the nerve fibre is as follows: Current from a neighbouring active region depolarizes the membrane by spreading along the cable structure of the fibre. As a result of this depolarization sodium current is allowed to flow (adapted with minor changes from Hodgkin and Huxley, 1952). Thus, both in the case of the space clamped membrane action potential and in normal propagation, the ionic currents of the spike proper are preceded by an outward current, a spatial displacement of ions.

The hypothesis deals with the events initiated by this early outward current which lead to the excited state.

Proposition 2. Outward exciting current moves potassium from the axoplasm into the membrane phase. (a) Some charged particles must carry the early outward current even if they only move on to and off a high capacity structure of some sort in the membrane phase rather than through the dielectric. The most likely candidate for the role of the particle which normally carries this current into the membrane cathodally is potassium derived from the superficial exoplasm under the membrane.

(a1) Potassium does move toward a cathode in electrically polarized nerve (Solomon and Tobias, 1951; Hodgkin and Keynes, 1953) and moves there

with a mobility close to that in water (Hodgkin and Keynes, 1953).

(a 2) In spite of the relatively high mobility of axoplasmic potassium, mobility alone would favour H+ ($\lambda c = 349.8$) over K+ ($\lambda c = 73.5$) ions for carrying the current by a factor close to 5 if the mobilities in water are used for the calculation. However, concentration difference in the axoplasm gives K+ an advantage over H+ of about 10^6 times. Therefore, considering only mobility and concentration, potassium will supply an overwhelming number of highly mobile axoplasmic current carriers.

(a 3) Potassium efflux at a cathode approximately accounts for the outward current there (Hodgkin and Huxley, 1952).

(a 4) The mean potassium transference number for current flowing out of the squid axon perfused with $(K^{42})_2SO_4$ is about 1.09 (Mullins and Brinley, 1964).

Proposition 3. There is calcium in the membrane phase: (a) There are two pieces of direct evidence.

(a 1) Micro-incineration followed by microscopy of myelinated nerve shows the calcium to be in the myelin and not in the axoplasm (Scott, 1940). To the extent that myelin is a repeating array of cell-membrane material (Robertson, 1961), this shows calcium to be present in the cell membrane. Calcium is also found by this technique to be concentrated at the surface of the neurone soma (Scott, 1940).

(a 2)Human erythrocyte ghosts contain about 1×10^{-4} m. equiv. of calcium/mg dry material (Mikulecky). To the extent that the red cell ghost is cell membrane this also shows calcium to be present there.

(b) There is a large amount of indirect evidence. A few examples follow.

(b 1) Calcium is needed for repair of membrane rupture in many cells (Heilbrunn, 1952) and decline of the injury potential in excised muscle is

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accelerated by high calcium in the medium (Stembach, 1933). Both effects presumably are due to the role of calcium in reconstituting membrane structure.

(b 2) Calcium decreases the width of the dense, double-edged membranes at the axon surface (Geren and Schmitt, 1954) as well as of binomolecular leaflets of phospholipids extracted from neural tissue and dispersed in water (Palmer and Schmitt, 1941).

(b 3) The numerous effects of calcium on excitability (Brink, 1954), on permeability (Heilbrunn, 1952), and on osmotic haemolysis (Moskowitz and Calvin, 1952) also indicate that it reacts with membrane components to modify structure.

(b 4) Effects of calcium on phospholipid monolayers will be noted in Proposition 5.

Proposition 4. There is phosphatidylserine in the membrane:

(a) The erythrocyte ghost contains phosphatidylserine (Kirschner, 1957; Dodge, 1963; Prankerd, 1961).

(b) Myelin contains phosphatidylserine (Brante, 1949).

Proposition 5. The calcium in the membrane is, in part at least, combined with the phosphate and/or carboxyl groups of membrane phosphatidylserine:

(a) There is no direct evidence for the intact cell.

(b) There is impressive evidence from studies on monolayers.

(b 1) If monolayers of purified phosphatidylserine (PS), phosphatidylcholine (PC), or phosphatidylethanolamine (PE) are made on a Langmuir trough, adding calcium to the aqueous hypophase condenses the PS film, but has no significant effect on the force-area diagram of films made either of lecithin (PC) or of cephalin (PE). This is true over a range of several units on either side of pH 7, therefore including physiological

pH values (Rojas and Tobias, 1964).

(b 2)   A Geiger-Muller counter suspended over a Langmuir trough containing radioactive calcium in the hypophase solution shows that spreading a monolayer of PS increases calcium-45 in the surface whereas monolayers of PC or PE have no such effect (Rojas and Tobias, 1964).

(b 3)   A membrane model made of phospholipid impregnated millipore increases its transmembrane resistance on addition of calcium to the ambient medium (Tobias, Agin and Pawlowski, 1962), but only if the phospholipid is PS, not if it is either PC or PE (Nash and Tobias, 1964).

These observations are understandable in terms of the acid-base properties of the three phospholipids: PC and PE are nearly electrically neutral around pH 7 whereas PS, having three ionogenic groups, a positively charged amino, and two negatively charged acidic groups, phosphate and carboxyl will, at pH 7, have at least one negatively charged group available for reaction with cations (Oncley, 1959).

Proposition 6. Excitation mobilizes the calcium of the membrane. Potassium, moved into the membrane by the exciting current, is the agent by means of which this mobilization is produced (continued in proposition 7):

(a)   Excitation increases entry of calcium into and egress from squid axons and muscle (Hodgkin and Keynes, 1957; Bianchi, 1961). Stimulation of the Elodea cell permits calcium to move into the vacuole (Mazia and Clark, 1936).

(b)   Adding potassium to the outside medium increases movement of calcium into and out of muscle (Bianchi, 1961).

(c)   The antagonistic actions of potassium with reference to calcium on electrophysiological properties, permeability, and structure of cells and of various colloidal systems also support the view that potassium can displace calcium.

Proposition 7. The calcium, so displaced by potassium, is displaced from membrane phosphatidylserine polar groups:

(a) Here it is possible to say that potassium can displace calcium from myelin and from phosphatidylserine. Direct data on cells are lacking.

(a 1) Monovalent cations displace calcium from bovine nerve myelin in the order lithium = sodium < potassium < rubidium < caesium (Leitch and Tobias, unpublished observations).

(a 2) The condensation of a monolayer of PS due to absorption of calcium from the hypophase is prevented by potassium (Rojas and Tobias, 1964)

(a 3) Radioactive calcium adsorbed to a PS monolayer is displaced by potassium (Rojas and Tobias, 1964).

(a 4) The rise in resistance produced in the phospholipid membrane model by addition of calcium is reversed by potassium chloride (Tobias, Agin and Pawlowski, 1962; Nash and Tobias, 1964).

(a 5) Direct chemical analysis (flame photometry) shows that calcium taken up by the phospholipid membrane model can be displaced by potassium, and the replacement obeys the rules of ion exchange phenomena (Mikulecky, 1963).

Proposition 8. These events, cathodally produced potassium intrusion and calcium displacement from phosphatidylserine polar groups, are followed by increased water entry into the membrane phase.

(a) Many experiments show that water content of whole cells is increased by adding K+ ions to the medium and is decreased by Ca++(see bibliography in Rojas and Tobias, 1964). There is also evidence that water content of axons is increased during activity (Tobias and Nelson, 1959; Tobias, 1951).

(b) In electrically polarized axons the cathodal region swells, increases in transparency, and becomes soft and sticky (Tobias, 1951; Tobias,

1952), these effects being ascribable to water migrating cathodally.

(c) In electrically polarized axons specific gravity of the axoplasm decreases cathodally coincidentally with the swelling. This is also ascribable to water migrating cathodally (Tobias, 1960).

(d) As mentioned under Proposition 3, X-ray diffraction shows that potassium increases and calcium decreases lamellar spacing of nerve myelin lipid extracts dispersed in water (Palmer and Schmitt, 1941), and calcium decreases the spacing of squid axon surface lamelae (Geren and Schmitt, 1954), both effects presumably being due to hydration changes.

(e) A carboxylic acid membrane, made from polyvinylmethylether and polyacrylonitrile plus vinylchloride, shrinks and loses from a half to a third of its water on going from the potassium to the calcium state (Gregor and Wetstone, 1956).

(f) Recent experiments on the phospholipid membrane model show that potassium increases and calcium decreases water wettability, water content, and water permeability of the model (Tobias, Agin, and Pawlowski, 1962; Leitch and Tobias, in the press.)

(g) The water content of myelin extracted from bovine nerve is increased by potassium and decreased by calcium (Leitch and Tobias, unpublished observations).

(h) Myelin in water forms an oil-in-water emulsion in the presence of potassium and a water-in-oil emulsion in the presence of calcium (Wolman and Wiener, 1963).

(i) The water content of red cell ghost fragments is increased by potassium and is decreased by calcium (Mikulecky, unpublished observations).

Proposition 9. Membrane protein contributes to the structural deformation of the membrane which is permitted by calcium displacement and started by water intrusion:

(a) There is one bit of direct evidence which suggests, though it does not prove, that protein may participate directly in the genesis of the change permitting the ion fluxes of activity. Protease injected into the axoplasm of the squid giant axon destroys excitability without interfering with the resting potential (Rojas and Luxoro, 1963). Such persistence of the resting potential indicates that potassium and sodium segregation and membrane resistance and capacity are maintained. Phospholipases, on the other hand, which also produce inexcitability, in addition cause the resting potential, resistance and capacity to fall to zero (Tobias, 1960). These findings suggest that integrity of the phospholipid in the cell membrane is mandatory for the high resistance and capacity which make the ion segregations and resting potential possible, and that protein is somehow involved in a reversible modification of the orientation, packing or structure of the phospholipid to permit the ion fluxes of activity (Tobias and Nelson, 1959; Tobias, 1958). An interdependence of lipid and protein geometry in the axon membrane is strongly indicated by electronmicroscopically demonstrable changes in the membrane protein component after phospholipid hydrolysis (Tobias, 1958; Tobias, Agin, and Pawlowski, 1962). This change would, according to the present view, co-operate with calcium displacement and increased hydration in producing the shift of membrane structure from the resting to the active state.

(b) Squid axon ATPase activity is concentrated in the sheath region, not in the axoplasm (Libet, 1948). One wonders if this enzyme may be a contractile protein associated with the membrane and capable of deforming it, since certain nerves have been shown to change surface contour and to shorten reversibly with activity (Kayushin and Tyudkovakaya, 1955; Bryant and Tobias, 1955; Sten-Knudson and Lettvin, 1962).

Conclusion

Displacement of membrane calcium by potassium (ion exchange event), increased membrane hydration (hydrokinetic event) and membrane protein deformation (proteokinetic event) underlie the reversible change in membrane structure (primarily phospholipid geometry) which expresses itself as: (1) a fall in resistance with (2) increased transmembrane fluxes of sodium and potassium, both being major signs of the excited state in nerve cells.

Additional Points and Queries

Propositions 2, 6 and 7. (a) Why does axoplasmic potassium not diffuse into the membrane phase to displace calcium and produce the excited state continuously? Why must it be driven into the membrane as an outward current?

To keep potassium out of the membrane phase is probably a function of the resting potential (Tobias, 1964). Assuming that only a few potassium ions need diffuse out of the axoplasm, leaving their non-diffusible anions behind, to produce the resting potential, these few do not displace enough membrane calcium to shift the membrane to the active state, and the resting potential so generated, being close to the potassium equilibrium potential, prevents further movement of axoplasmic potassium into the membrane phase.

This concept is supported by a recent experiment (Narahashi, 1963): Normally, both axons and muscle cells become inexcitable if the resting potential is kept low. However, if internal potassium is reduced sufficiently, then axons are excitable even at zero resting potential. In terms of the present hypothesis this would be explained by arguing that when potassium is present internally in normal concentration, then a large resting potential is necessary to keep this potassium out of the membrane (the equilibrium potential is high) so that the high resting impedance of the

membrane can be maintained until the exciting stimulus is applied. If, however, there is little or no potassium in the axoplasm, then little or no resting potential is needed to keep it out of the membrane phase (the equilibrium potential is low), and in this condition the system can be excited from a low or zero resting potential level.

(b) How much potassium is moved into the membrane phase by the early, exciting, outward current?

An average of about one potassium ion per 45,000 sq. $\overset{\circ}{A}$ of membrane area can be calculated as the amount of potassium which is moved into the membrane catelectrotonically by the time threshold is reached. This is computed from the action current at the frog node of Ranvier (Tobias, 1952), from squid axon membrane capacity (Hodgkin and Huxley, 1952) and from the number of coulombs of outward current per cm^2 needed to excite the squid axon (Hodgkin and Huxley, 1952). Since, however, if 1 per cent of the membrane area became freely conducting the resistance as a whole would fall to about $0.002 \approx cm^2$, and since membrane capacity changes little with activity, it can be argued that perhaps as little as 0.001 per cent of the area is involved. Thus, considering the membrane to be 100 $\overset{\circ}{A}$ thick, and if only 0.001 per cent of the area were involved, the concentration change due to the current carrying K into this volume of membrane would be about 37 millimolar by the time threshold is reached. It has been found that something between 10 and 100 millimolar potassium reverses the effect of 1 mM calcium on the phospholipid membrane model and displaces calcium from the phosphatidylserine monolayer (Rojas and Tobias, 1964). For an internal concentration of potassium of 0.1 M the number of potassium ions needed for the foregoing change could be supplied from a sub-membrane layer about 5 $\overset{\circ}{A}$ thick.

(c) If depolarization moves potassium into the membrane phase and this

leads to structural dispersion with a fall in resistance, what keeps membrane resistance relatively high during the prolonged plateau of depolarization normally shown by heart muscle (Weidmann, 1951), and by nerve treated with tetraethylammonium or certain metal ions (Tasaki, 1959)?

Here it is suggested that after potassium displaces calcium from phospholipid during excitation in the presence of TEA or nickel, then, during recovery, these unusual ions in turn displace potassium and occupy the sites which would normally be reoccupied by calcium. If they bind to phospholipid more tightly than calcium does, then they would tend to stabilize the membrane structure in the high resistance state, and prevent potassium egress, thus delaying repolarization. As time passes, however, the persisting low potential will eventually permit enough potassium to accumulate in the membrane to displace the binder ion. When this finally occurs the potassium will flow out and repolarize the cell. In this way the persistence of relatively high resistance even in the presence of depolarization could be accounted for. Experiments on the quantity of outflowing current required to excite under these circumstances and on tenacity of TEA and nickel binding to phospholipid as compared to that for calcium could be done fairly easily and would clarify this problem. What substance normally present in heart muscle might produce this effect is not apparent, but the suggestion that such a substance exists may stimulate interesting experiments.

(d) Since neither the phospholipid membrane model nor the monolayers used so far show any ability to distinguish potassium from sodium, what protects the phospholipid-calcium complex from external sodium?

No satisfactory answer is at hand. It has already been suggested here that it is probably the resting potential which protects the resting membrane from potassium, but we do not know what protects it from sodium. Failure to answer this question, however, does not weaken the hypothesis. It does

emphasize the fact that one still does not know how cells distinguish sodium from potassium.

(e) Perfusion of the squid axon with low-potassium or potassium-free solution does not prevent excitation (Narahaski, 1963). How can this be reconciled with the argument of the hypothesis that it is catelectrotonic injection of axoplasmic potassium into the membrane which displaces calcium to start the structural changes which permit the fall in resistance to ion fluxes?

First of all, as internal potassium is decreased, an increasing amount of depolarization is needed for the membrane resistance to fall to a given value (Narahaski, 1963). Since a change in membrane resistance is probably our best evidence of a change in structural state, this finding supports the hypothesis. Thus, in terms of the hypothesis, the less potassium there is inside the axon the more depolarization should be required to permit and enough potassium to enter the membrane phase to shift it to the low-resistance state.

Secondly, it is not argued that K+ is the only cation which can be moved into the membrane phase from inside to displace calcium from negatively charged sites there. We know that sodium is about as effective as potassium in displacing calcium from phosphatidylserine (Rojas and Tobias, 1964). What is argued is that when potassium is the dominant internal cation, then it is the ion which is moved into the membrane to produce this effect. It is, so to speak, the ion which the cell normally uses to trigger excitation. In the experiments quoted (Narahaski, 1963) there always had to be high external sodium. This sodium would be expected to leak continuously into the inside perfusate and under these circumstances almost surely would the cation be moved outward by the outwardly directed exciting current to displace calcium from the membrane structure. Thus, the potassium-free perfusion experi-

ments do not pose a serious problem for the hypothesis.

Proposition 3. It becomes ever clearer that an explicit demonstration is required of what calcium is associated within the cell membrane. The evidence for certain reasonable assumptions has been given, and the possibility of combination with nucleic acids has also been investigated (Rudenberg and Tobias, 1960), but there are no objective experimental data yet available which apportion membrane calcium among the several likely membrane components.

Proposition 8. Since cells are highly permeable to water, that is, if there is a relatively high partition co-efficient for water in the membrane, why should increased membrane hydration increase permeability for ions?

The fact is that the resting cell membrane may not be significantly hydrated in spite of high water permeability. Thus, the normal red cell surface is relatively hydrophobic (Mudd and Mudd, 1926), the denuded Arbacia egg in calcium chloride coalesces more readily with oil than when it is in potassium chloride (Kopac, 1940), myelin and red cell ghosts contain less water in the presence of calcium than in potassium (Leitch and Tobias, unpublished observations; Mikulecky, unpublished observations), myelin forms a water-in-oil emulsion in the presence of calcium and an oil-in-water emulsion in the presence of potassium (Wolman and Wiener, 1963), and for many resting cells a high oil : water partition coefficient favours penetration. Therefore it would appear that water can penetrate what may be a water-poor cell membrane phase just as it can to a degree penetrate the hydrophobic part of evaporation retarding monolayers used to conserve water in reservoirs and lakes (LaMer, 1962). Presumably, however, ions could not penetrate significantly by the same pathway and would require increased hydration with formation of water-filled conduits.

Clearly experiments need to be done to determine if axon membranes do

in fact become more hydrophilic when excited. Some starts have been made which suggest that water entry into axons increases during activity (Tobias and Nelson, 1959; Tobias, 1951; Tobias, 1960; Bryant and Tobias, 1955).

Proposition 9. The nature and role of protein in membrane excitation are almost completely unexplored (Ungar and Romano, 1958; Luxoro, Rojas and Wittig, 1963). Recent work on a membrane model made of a bimolecular lipid leaflet in water (Mueller and Rudin, 1963) suggests that excitability is conferred on the system only after addition of a specific protein. Even though ATPase was long ago shown to be concentrated in the axonal sheath structure (Libit, 1948), there are not yet any reports of serious attempts to find a contractile protein in nerve cell somata or fibres.

Magnitude of the Transmembrane Potential

It has been argued (Tobias, 1952) that the fraction of the resting potential which still exists after threshold is reached at about 15 mV depolarization could do a significant amount of work on membrane components which had been released from mechanical restraint by unbinding of calcium and intrusion of water and which were exposed to this voltage gradient. Thus, it was calculated that a voltage of 50 mV across 100 $\overset{o}{A}$ (50,000 V/cm) could move a molecule the size of serum albumin through a distance of 100 $\overset{c}{A}$ in about 3×10^{-4} msec. This figure represents maximum speed assuming application of the full voltage and mobility as in water. The velocity is so great, however, compared with the local duration of the nerve impulse, that even with considerable attenuation, say, 1×10^3 or so, such a phenomenon might still play a significant part in altering surface ultrastructure. In addition, it might not be necessary to produce appreciable translation of the liberated membrane component; only a change in orientation as in alignment or disalignment of a dipole might be required. If such a device were operative, then one would add electrokinesis to ion exchange, hydro-

kinesis and proteokinesis as basic components of the excitation process. The recent finding that a persistent zero resting potential can be tolerated without immediate disastrous consequences (Narahaski, 1963) de-emphasizes this concept somewhat. Nevertheless, such steep voltage gradients may have important consequences in other contexts.

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MACROMOLECULAR CONFORMATION CHANGES AS POSSIBLE
INFORMATION PROCESSING MECHANISMS*

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There is much interest in those biochemical changes which may correspond to learning or training in lower animal forms and also those which may correspond to the storage and retrieval of so-called memory. In some cases, it is assumed that the formation of new synapses may be a prerequisite for either or both of these processes (Roberts, 1963). Other studies have focused on the potential importance of RNA synthesis in learning and maintenance of training both in planaria and in rats (Hyden, 1962; Corning and John, 1961), or the role of protein in the maintenance of memory (Dingman and Sporn, 1961). Others have postulated that both the consolidation and retrieval of information in the brain may involve antigen-antibody type reactions (Silverstein, 1964).

The present report reviews the results of studies on how human beings process information and then considers whether proteins have the properties required of "flip-flops" which might be critically involved in information processing and storage. It is to be emphasized that whereas "flip-flop" units might actually be appropriate for long term storage if memory is stored in a binary fashion, the present report is concerned principally with events involving one quarter second or less.

Resume of Studies of Information Processing in Humans.

Since 1954 a number of reports have been issued by the Control Systems Laboratory at the University of Illinois concerning the properties of man as an information transducer (Quastler and Wulff, 1955; Quastler, 1956; Augenstein, et al, 1956; Augenstein, 1956). In almost all cases these studies were concerned with the information processing capabilities of subjects performing well-learned tasks. Those findings which are most pertinent to the present discussion are briefly listed below.

1. Humans performing tasks such as piano playing, typewriting, and mental arithmetic (in all cases completely randomized texts were used), process information at a rate equivalent to 15-25 bits/sec. (Quastler and Wulff, 1955; Quastler, 1956; Quastler and Augenstein, manuscript in preparation). At least in piano playing there is evidence of a channel capacity of 22 bits/sec. so long as the uncertainty of each note is 4-6 bits (i. e., the random text was composed by selecting from 16-64 possible notes). Two studies in other laboratories indicate that some subjects can read a list of randomized words at a rate corresponding to 30-45 bits/sec. (Licklider, Stevens and Hayes, 1954; Pierce and Karlin, 1957). Most estimates associated with unnatural symbols or activities give much lower rates.

2. When subjects memorized a random text they could increase their speed of typing or piano playing by as much as a factor of 2 or 3. This indicates that responding does not impose the ultimate limitation.

3. Data acquisition (i. e., input) can also be ruled out as the rate limiting operation in determining the S's capacity. That is, not less than 10 and as much as 50 bits can be acquired via the visual pathway in times of the order of a twenty-fifth of a second (Augenstein, et al, 1956; Augenstein and Quastler, manuscript in preparation; Auerbach and Coriol, 1961). Further, it is well known that when people look at pictures, read

text, etc, the eyes are not constantly in motion, but rather there are gross movements only 4 or 5 times/sec. (e. g., Buswell, 1955; Handbook of Physiology, Vol. 2). Apparently, when "left to its own devices" the eye takes in information in a very short interval of 10-40 msec. and then remains refractory for about 200 msec.

4. Thus, data processing must be the operation which exerts the primary influence on a human's overall information processing capacity. In a number of experiments (e. g., scanning a column of randomized letters and numbers to find the first number, adding columns of numbers, typing random text, reconstructing a three-letter word from scrambled trigrams, etc.) the response times are obviously quantized (Augenstein, 1956; Augenstein and Quastler, manuscript in preparation). Although there is almost certain evidence of a 100 msec. periodicity, it appears that this is the predominant rather than the fundamental time quantum. The available reaction time data can all be adequately fit assuming that the fundamental period is actually 33 msec. and for some reason these tend to occur in clusters of three. However, it is to be emphasized that the data were all collected accurate to 10 msec. so that it would not have been possible to demonstrate fundamental periods of durations less than 25 msec. Thus, experiments now in progress--collecting data accurate to 1 msec.--must be completed before it can be determined whether the predominant 100-msec. periodicity may actually be composed of four 25-msec. periods, or five 20-msec. periods, or six 17-msec. periods...or perhaps even ten 10-msec. periods.

5. It was postulated previously that of the thirty 33-msec. unit acts available each second, four or five are used for the intake of large pulses of data and the remaining twenty to twenty-five are utilized for processing the 20-25 bits of information which are required for typing and piano playing.

Thus, according to those postulates, during each 33-msec. unit act a S presumably can make a yes-no decision or may acquire a large amount of data (Augenstein, 1956).

The strongest inferential evidence for these conclusions is that six subjects performing a variety of tasks all showed the same predominant 100 msec. periodicity. Since the tasks employed varied from simply scanning through a column of 00, 11, 00 etc. symbols to find the first 10 or 01 to reconstructing three letter words from scrambled trigrams, Ss would not be expected to all have the same associated periodicity unless the completion of all of these tasks involve a series of simple stereotyped decisions. That is, unless processing does involve a series of binary (or as discussed below--trinary) decisions, it would be anticipated that the duration of the unit act involved in data processing would be a function of the complexity of the basic decisions required to complete the task.

Reservations must be attached to the above model because two reports of information processing in excess of 30 bits/sec. have been reported: Licklider, et al (Licklider, Stevens and Hayes, 1954) and Pierce and Karlin (1957) found that Ss can read randomized words at a rate corresponding to 40 bits/sec. For the above model to encompass these results requires (a) simultaneous processing in parallel channels; or (b) the unit act should have a duration of 20 msec. or less; or (c) processing should be on a trinary basis where each decision accounts for 1.6 bits. Although these possibilities can not be further evaluated until experiments now in progress are completed, it seems worthwhile to consider possible molecular and/or cellular mechanisms which might account for the uniform periodicity pattern observed for all Ss in the tasks investigated.

Certainly values of 100 msec. or less would be too short for the synthesis of macromolecules such as proteins or nucleic acids--unless of course

all except one residue were already preformed and data processing simply requires adding a last sub-unit (Haurowitz, 1963). A value of 10-33 msec. would not be an unreasonable time for the turnover of coupled enzyme systems. In fact, the turnover rate for the Krebs cycle is approximately of this order of magnitude (Reiner, 1953). The values cited above would also be reasonable time constants for a network of cells such as might be involved in localized regions of the brain (Lindsley, 1958). Also, diffusion processes over distances of the dimensions of a cell might be expected to occur in such a time interval. Whether antigen-antibody type reactions occur in this length of time is not known.

Although the four processes mentioned above, all seem to be reasonable ones to associate with such time constants, sufficient information is not available to select for or against any of them. The fifth possibility considered below is that the duration of the unit act represents the time associated with conformation changes which can represent a yes-no decision; that is, the time constant for a macromolecular flip-flop.

The Flip-Flop Characteristics of Proteins

The ideal flip-flop--whether it be mechanical, electrical, or molecular--in a data processing system should have two distinguishable states, which are discrete and thermally stable: transitions between the two states should occur with high probability for an acceptable stimulus, and it should be possible to go between the states reversibly--otherwise new processing elements must be continually resynthesized. While conformation changes can be produced in nucleic acids, it seems unlikely that those produced by the stimuli available in the brain would be sufficiently unique to provide a basis for accurate information storage and retrieval. That is, nucleic acids have only four nucleotides so that there is considerable

repetition of linear sequences, and further, in the helical configuration there is presumably considerable delocalization of excitation (Augenstein, 1963) with the result that there may be many sites at which the energy in a stimulus might produce molecular changes. Proteins seem to be a much more likely class of molecules to use as flip-flop elements since they have 20 rather than 4 types of monomeric units. Thus, the remainder of this section is concerned not with whether proteins actually do behave as flip-flops in information processing, but rather with the question of whether they have the properties which would be required to serve such a function. As can be seen from the following resume of the effects of temperature and UV radiation on two enzymes, trypsin and ribonuclease (RNase) do indeed have most of the necessary properties required of flip-flops.

In an attempt to explain the very great sensitivity of enzymic activity to heat and radiations, one of us proposed a number of years ago the following weak-link hypothesis (Augenstein, 1957). It is well known that only a small fraction of the surface area of enzymes is actually involved in enzymic function. Thus, if the effects of various physical agents such as heat, radiation, or interfacial forces become localized in bonds responsible for maintaining the spatial integrity of those residues critical for function, biological activity could be destroyed. In particular, it was proposed that a cluster of individually weak bonds are responsible for maintaining the integrity of this so-called active center: this cluster includes one or two cystine residues and additional intramolecular bonds--probably hydrogen bonds. The weak link model further proposes that if only a fraction of the critical bonds are broken, reversible inactivation occurs whereas if the whole cluster is disrupted, such a large entropy change occurs that reactivation of the biological function is impossible. Using ultraviolet light (of sufficient energy to cause excitation to the lowest-lying optically-

allowed excited level) as the disrupting agent it has been possible to dem-
onstrate the following five predictions based upon this model.

A. With increasing doses of UV the disruption of cystine groups--
as evidenced by the appearance of SH groups which can be titered by p-chloro-
mercuribenzoate (pCMB)--is directly correlated with the loss of trypsin and
RNase activity (Augenstein and Ghiron, 1961). Analysis of the rate of ap-
pearance of SH groups shows that the various cystines in trypsin and RNase
have radiosensitivities that differ by as much as a factor of 10 (Augenstein
and Reilly, in press). With the exception of tryptophan the destruction
of the other constituent amino acids is too slow to account for any appre-
ciable fraction of photoinactivation in trypsin (Augenstein and Reilly, in
press).

B. Autoradiographic studies have shown that UV doses which will pro-
duce up to 75% destruction of enzymic activity in RNase disrupt one cys-
tine group almost to the exclusion of the other three (Augenstein and Grist,
1962).

C. The addition of a specific SH reagent (such as pCMB) to trypsin
subsequent to irradiation and prior to exposure to its substrate increases
the apparent amount of inactivation for a given dose of radiation: that
is, "tieing up" radiation-produced SH groups prevents reactivation of dam-
aged trypsin molecules when substrate is finally added (Augenstein and
Ghiron, 1961).

D. Irradiation at elevated temperatures or the addition of urea sub-
sequent to irradiation and prior to exposure to substrate increases the
amount of trypsin inactivation for a given dose of UV compared to the sit-
uation when irradiation is followed immediately by the addition of sub-
strate. The thermal coefficient of 2.2 kcal/mole. agrees precisely with
the values expected for the disruption of hydrogen bonds providing the

excitation of a proton via the torsional mode is an essential step in hydrogen bond disruption (Augenstein, Atchison, Grist and Mason, 1961).

E. The addition of substrate immediately after irradiation produces reactivation which can not be subsequently destroyed by urea. That is, if following irradiation substrate is added first followed by urea before assaying, the amount of inactivation is greatly reduced from that measured when only urea is added after irradiation prior to assay (Augenstein, 1959).

F. Appropriate measurements have not as yet been made to determine the actual time involved in protein conformation changes.

It can be seen from the above that UV does not produce random destruction in the two proteins studied, but rather preferential cystine disruption; and the damaged states produced in trypsin can be returned to an active configuration by interaction with substrate. Thus, not only is it possible to elicit two distinct conformations and to go between them reversibly, but titration with pCMB at various times after irradiation indicate that at pH 4.5 damaged trypsin molecules are stable at room temperature for at least a few hours. Nevertheless, the quantum yield for this process is not very high, being only approximately 1 in 60. Further, UV quanta are not available in the brain. Recent preliminary experiments, however, suggest that changes in proteins comparable to those produced by UV can perhaps be produced by electric fields and/or currents of the order of magnitude of those which exist across nerve cell membranes. For example, 100 mv across a 100 $\overset{\circ}{A}$ membrane corresponds to 10^5 v/cm (Hodgkin and Huxley, 1952). The reports that potentials of a few hundred v/cm can produce electroluminescence in anthracene indicates that these fields can cause excitation to at least low-lying excited levels (Gurnee, 1962).

Recently we have studied the effects of electric currents and/or fields on proteins in solution without other ions (not even buffers)

present. Passing even 1 mA or less produced a marked reduction in tryptic activity in 10-30 minutes. Further, the concentration and activity of samples taken at various points in the sample compartments vary considerably. With a 30 V drop across 20 cm. of solution, when the current is first turned on there is a movement of protein away from the positive toward the negative electrode which reaches a peak in about 10 minutes. During this time, however, there is relatively little loss in the absolute amount of activity or in the distribution of activity within the sample compartment. Appreciable loss of enzymic activity is observed after about 30 minutes.

These results indicate that at least with current flows and potential fields comparable to or even less than those which exist in the brain it is possible to modify trypsin sufficiently to destroy its enzymic activity. Additional experiments now in progress should indicate whether inactivation requires current flow or simply excitation within a high potential field such as may exist at the membranes separating the compartments. Much more detailed experiments are now required to determine whether the inactivation produced involves sufficiently unique conformation changes and in sufficiently high yields so that proteins may serve as ideal flip-flops.

If further studies do show that electric fields and/or currents can produce the desired changes in proteins in solutions or at interfaces, then detailed studies on nerve cells are needed to determine whether proteins may indeed perform their functions by serving as switching elements. That is, conformation changes may drastically alter conductivity in affected elements (Blyumenfeld and Kalmanson, 1958). Obviously the possibility should also be investigated that flip-flop operations in individual elements of assemblages of macromolecules may provide memory storage comparable to the binary elements utilized in digital computers.

Processing on a trinary basis--as briefly discussed above--could be accomplished by binary flip-flops arranged in such a fashion that neighboring elements can exert a modifying effect on the behavior of adjacent elements (Pitts and McCulloch, 1947). Alternatively, trinary operations could be accounted for by molecules which can exist in three states. Actually UV treatment results in three classes of trypsin molecules (i. e., active, damaged and inactive); however, the desirable property of reversibility applies to only two of the states so produced (Augenstein and Ghiron, 1961; Augenstein, 1959).

It is to be emphasized that the present report is directed at the question of how proteins might function as elements in a data processing system in humans. Quite obviously the postulates concerning the nature of information processing in humans require further extensive testing; at the moment their main virtue is that they suggest good experiments. Similarly, the encouraging studies on the effects of electrical currents and/or fields on proteins are in the preliminary stages. Even if the model proposing that man breaks all decisions down to a series of yes-no decisions is found to be true and if electric currents can produce unique, stable, conformation changes which are reversible between two states of a protein, it will take much more work to bridge the gap between the question of whether proteins could serve the anticipated function in the nervous system and whether they actually do.

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ANTIBODY FORMATION AND IMMUNOLOGICAL MEMORY

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Though immunology deals with complex behavior and with the role of macromolecules engaged in that behavior, I am afraid that my subject is only marginally relevant to the other discussions of this conference. I shall therefore try to make my exposition brief. Certain terms occur in immunological usage, such as "memory" and "recognition," which in their original meaning refer to phenomena linked to the function of the central nervous system. The analogies between the immune system and the central nervous system appear to be quite superficial, however, and it would seem far-fetched to suggest functional similarities. All the same, it might be worthwhile to take a brief look at immunology and at the mechanisms known to be at work in this biological system.

The main phenomenon of immunology is that an animal, when exposed to an antigen, reacts by exhibiting an immunological response specifically directed against that antigen. This phenomenon appears to be restricted to vertebrates; it has not been demonstrated in other animals or plants. Among the specific immunological responses, the best known are (a) the formation of specific antibody to a given antigen, (b) the establishment of specific sensitivity to a given antigen, (c) the rejection of a skin graft or a tissue transplant from another individual of the same species, and (d) the establishment of specific immunity to a disease when the antigen represents a pathogenic virus or bacterium. The ways in which these four types of specific immunological responses are related are not exactly known. The type of response that appears most readily subject to analysis is antibody formation.

I shall consider three elements in antibody formation - first, the antigen, secondly, the immune system in the body that responds to the anti-genic stimulus, and thirdly, the antibody molecules produced.

1. The antigen can be one of an immense number of substances. In fact, practically all macromolecular substances or structures obtained from the organic world are antigens. They can be proteins, lipiproteins, carbo-hydrates or nucleic acids. Even small inorganic molecules or groups can function as antigens when attached to macromolecular carriers. Moreover, artificially synthesized molecules that have never before existed in our universe can function as antigens. It has therefore often been stated that the number of different antigens is infinite. Only a finite, rather small number of antigens has even been studied, however, and even among the anti-gens studied, so-called "cross-reactions" have frequently been noted. All the same, it is clear that an animal can respond to each of an enormous num-ber of different antigenic stimuli by the production of specific antibodies and it seems likely that the number of antibodies of different specificities that one animal is potentially capable of making is of the order of one mil-lion or more.

2. The organ or the "immune system" which deals with immunological stimuli and responses in an animal is the so-called lymphoid-macrophage sys-tem. This comprises organs such as the spleen, the thymus, the lymph nodes and lymphatic vessels, the appendix, and other lymphoid tissue scattered widely throughout the body. The main cells in this system are lymphocytes and macrophages. The lymphocytes are not capable of phagocytosis. The macrophages are phagocytic cells associated with areas where lymphocytes occur in large concentrations. The total weight of the immune system of cells in an adult human is 1 to $1\frac{1}{2}$ kilograms and the number of lymphocytes participating in the system at any one time is of the order of 10^{12} cells.

Among these lymphocytes, various classes and stages of differentiation can be cytologically distinguished but neither the sequence in which differentiation takes place nor the lifetime of the cells is as yet fully known. The majority of the cells are so-called small lymphocytes. Some of these cells appear to have a half life of a few days or less, whereas others have half lives of 100 days or more, and there are even indications that some may circulate in the body for more than 10 years. Small lymphocytes may be derivatives or precursors of large lymphocytes. Antibody formation by single cells has been demonstrated. Most of these cells were large lymphocytes or plasma cells. Such a cell can release antibody at the rate of several thousand molecules per second. It has recently been found that no antigen molecules appear to be present in the lymphocytes that produce antibody specifically directed against this antigen (Nossal, in press). The antigen molecules which constitute the antigenic stimulus are taken up by macrophages and it is widely believed that these macrophages then transmit a signal to the lymphocytes which induces these cells to specific antibody formation. This sequence of events is not proven, however, and it may still be that antigen can stimulate lymphocytes directly without passing into intervening macrophages.

3. The antibody molecules that are synthesized in response to an antigenic stimulus belong to a large number of different classes of protein molecules, all of which are called "immuno-globulins" or "gamma-globulins." Antibodies are physico-chemically indistinguishable from the gamma-globulins that normally circulate in the blood in a concentration of about 5×10^{16} molecules per milliliter. It is believed that all of these are antibody molecules even though the specific antibody properties of a gamma-globulin cannot be shown unless the antigen against which it is directed is known. Most antibody molecules possess two specific sites - the so-called

"combining sites." These two sites have identical specificity and are situated at two opposite ends of the globulin molecule. Most immunologists believe that a specific site is made up of a small number of amino acid residues and that differences in specificity are due to spacial configurations which are determined by differences in amino acids and amino acid sequence. Even antibody molecules of the same specificity, however, belong in many different molecular classes; some (19S) have a molecular weight of close to one million, whereas others (7S) have a molecular weight of 160,000. Among the latter, different classes can be distinguished that travel with different electrophoretic speeds in agar-gel. There are also differences with respect to the firmness with which antibody molecules can combine with the corresponding antigens. The 19S antibody molecules contain about 10% carbohydrate, whereas the 7S molecules have only about 2-3% carbohydrate. The structure of the 7S antibody molecule has, during recent years, been the subject of intensive studies. It is now known that this molecule contains four distinct polypeptide chains, two longer H (or A) chains and two shorter L (or B) chains, inter-connected by disulfide bonds. Experiments have shown that isolated H chains retain a certain degree of specificity towards the antigen and it is therefore believed that the major contribution to the combining site is due to a series of amino acids situated in the H chain. In humans, there seem to be at least 20 different types of L chains that can be present in antibody molecules of one specificity.

It is clear that it would be of great interest to know in which way the immune system functions or, more simply, in which way an antigenic stimulus leads to antibody formation. This knowledge would have a great impact on practical procedures of specific immunization against disease and on the attainment of successful organ-transplantation in replacement of deficient organs, for which the surgeons are now ready. It would also solve a major

biological paradox which seems to be inherent in the capability of cells to produce protein molecules that are specifically directed against antigens which the body may never have encountered before. In view of the immense number of different antigens to which the system can respond, most immunologists have so far rejected the idea that all different antibody specificities could have been coded for in the DNA of the population of lymphocytes prior to the arrival of the antigen. On the other hand, it seems clear that antibody molecules are synthesized in the same way as enzymes and other protein molecules, namely by assembly of polypeptide chains on ribosomes that obtain their information from messenger RNA.

We are confronted with a process of "learning." The individual has the innate potentiality of forming innumerable types of antibodies but will only make those that are directed against the antigens to which he becomes exposed during his lifetime. He will not transmit this "learning" to his offspring. In this sense, the immune system is perhaps the only extensive physiological system whose function bears a similarity to the phenomenon of learning in the central nervous system. In both cases, the amount of information that the body can potentially learn to express is perhaps so large that it cannot be coded into the DNA of the zygote.

When an individual has once reacted immunologically to an antigen, he is no longer the same with respect to that antigen. When confronted with this same antigen a second time or on subsequent occasions, the immune system will react more quickly and more abundantly. In antibody formation, this is called the "booster" response. This is a well-known phenomenon in immunology and is the reason why certain immunizations against infectious disease work better when repeated by so-called "recall" injections. In transplantation this effect is seen in the so-called "second-set" rejection, the phenomenon that the rejection of a second transplant from a donor occurs

much more quickly than the rejection of the first transplant from that same donor. It is also evident in acquired sensitivites such as hay fever, asthma, and other allergies. In all these cases then, the immune system tends to react more effectively when a specific antigenic stimulus is repeated. This again bears a certain resemblance to learning by repeated application. The term "immunological memory" is used to describe the fact that in many instances the immune system retains its capacity to react with increased efficiency to a given antigen during many years after the last contact with that antigen. Some extreme instances of this memory phenomenon are well known. Thus, when a measles epidemic broke out on the Faroe Islands in 1846 practically all of about 7,000 inhabitants of these islands contracted measles within six months. The only exceptions were a few persons who were over 70 years of age, and it was shown (Panum, 1847) that these persons had had measles in the preceding epidemic that had taken place on these islands in 1781. The immune system of these individuals had retained the memory of this earlier occurrence. Similar instances are known in influenza where successive epidemics are due to viruses that have different antigenic specificity. Some old people still contain demonstrable antibodies against a type of virus that is believed to have been prevalent in the influenza pandemic of 1889-1890. Experimentally it can also be shown that certain antigens produce either a response that lasts for many years or that leaves the animal in a state in which he will quickly respond to a secondary exposure.

Various theories have been proposed to explain the mechanism of antibody formation. For many years it was thought by most immunologists that antibody molecules were formed directly in contact with antigen molecules, in such a way that the newly synthesized polypeptide chain of a gamma-globulin molecule would fold so as to gain a tertiary structure complementary to

the adjacent antigen molecule. This theory has to be discarded, however,
since the formation of antibody molecules in a lymphocyte must proceed at
something like 100,000 sites simultaneously in the absence of demonstrable
antigen. Many immunologists still would like to believe that the antigen
plays an "instructive" role in antibody formation, for instance, by a mech-
anism permitting the antigen to direct the production of a specific RNA,
perhaps in the macrophages. This RNA might then be transferred from the
macrophage to a lymphocyte in which it might be self-replicating and serve
as messenger RNA for the production of specific antibody molecules. It
seems very difficult, however, to understand, first, how an antigen could
serve as a template for an RNA nucleotide sequence and, secondly, could
determine that sequence in such a way that the ensuing protein molecule
would possess complementarity to the antigen. There is another question
that should be considered in this connection and that is the phenomenon
that has been termed "self-recognition" or "recognition." The immune sys-
tem does not normally produce antibodies against the components of the in-
dividual itself, though these components may be perfectly good antigens
when injected into another individual. The immune system must be able to
distinguish between antigens that occur in the individual itself and anti-
gens that are foreign. When an antigen is injected into an individual,
the first decision that must therefore be made is whether this antigen is
"self" or "foreign." There must be a recognition system which precedes
antibody formation. In recent studies of the phagocytosis of antigen by
macrophages associated with the lymphoid tissue, Nossal (in press) has
shown that foreign antigens are immediately taken up by these macrophages,
whereas components of the animal itself (taken out of the animal, labeled
and reinjected) are not taken up by the macrophages. The only exceptions to
this rule were the normal gamma globulins of the individual itself. It

might thus well be that the macrophages recognize only the gamma globulins of the individual himself, and that these "normal" gamma globulins are the molecules that recognize the antigen and make the primary distinction between foreign antigens and self antigens, by attaching only to those that are foreign. As I said before, the spontaneous pool of "normal" gamma globulins in blood is of the order of 5×10^{16} molecules per milliliter. If we assume that this large amount of molecules comprises a million or more different antibody specificities representing all antibodies that the individual can make and directed against foreign antigens that may not so far have made their appearance in the individual, then this pool of gamma globulins could constitute the primary recognition system and the antigen might function by selecting preformed molecules and, via uptake into the macrophages, by presenting these molecules to the immune system for a more rapid rate of production. This scheme constitutes one of the so-called "selective" theories of antibody formation which are based on the idea that the organism acquires no new knowledge by the exposure to antigen but has already spontaneously created samples of antibodies of all possible specificities in order that the "fitting" ones can be selected as circumstances may dictate. In considering learning by the central nervous system, this would correspond to the idea that we can never truly learn anything new. As pointed out by Socrates (375 B. C.) and Kierkegaard (1844), understanding must be preceded by recognition, and all learning, therefore, must consist of a "recollection" of knowledge already present in the soul.

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A CRITICAL APPRAISAL OF THE ROLE OF RNA
IN INFORMATION STORAGE IN THE NERVOUS SYSTEM

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Over the years scientists have been intrigued with the problem of how new behavioral experience can be permanently coded within the nervous system. Each generation feels that it has the solution in the techniques which were appropriate for solution of other major problems of its era. In the present time, the advances of molecular biology have contributed much to our understanding of how the genetic potential of a bacterial cell is translated into the enzymes which define its metabolic state. "Messenger" ribonucleic acid has evolved as an important concept because it provides an explanation of how the code in DNA may be carried to the ribosome by a messenger RNA and will be decoded there by small molecular weight transfer RNA's each carrying a specific amino acid (Monod and Jacob, 1961). Since the messenger RNA's possess significant information content, it has been proposed by several investigators as the storage site for the memory process in the nervous system (Hyden; Schmitt, 1962). Each new memory trace would be defined by a new RNA macromolecule, and this molecule would persist as long as the memory trace persisted. Since many memories last for many years, this RNA would be expected to endure for this period. However, in the mammalian cells examined to date messenger RNA does not appear to be this permanent (Penman, Scherrer, Becker, and Darnell, 1963). Since the neuron is a cell specialized for impulse transmission, and since RNA is not known to play any direct role in such transmission, it may only function indirectly by directing the synthesis of those substances that do participate in intercellular communication. If information transfer and storage in the nervous system is a communal response determined by a complex

of interacting cells, it seems unreasonable that any single molecule could define the overall process. Even in bacteria where the cells are isolated units responding individually to the environment, RNA is only one link in the bacterial response to an altered environment which will include the interaction of the new external environment with the cell membrane, its transport and energy systems, with the genetic potential and protein synthesizing factories of the cell, and with the numerous control processes which permit the most economical cellular expression.

We shall review the role of RNA in information storage in the nervous system to ascertain whether it does, in fact, serve as the specific molecular counterpart of memory storage. Our aim is to examine the evidence in a way which will permit a better understanding of macromolecular and neuronal interactions and which may indicate a meaningful experimental approach to the complexities of memory processes in the CNS.

In recent years several experiments have examined the possible role of RNA in "learning and memory." The conclusions have been drawn that treatment with ribonuclease alters the retention of conditioned responses in re-
and John
generated planarians (Corning,/1961), that the structural analogues of purine bases affects the maze learning in rats (Dingman and Sporn, 1961; Chamberlain, Rothschild, and Gerard, 1963), that learning situations produce specific alterations in the base ratios of neuronal RNA (Hyden and Egyhazi, 1962), that tricyanoaminopropene increases neural RNA and promotes learning (Chamberlain, et al., 1963; Hyden and Hartelius, 1948), and that RNA administered to rats shortens the latency of acquisition and prolongs the period before extinction of a conditioned avoidance response (Cook, Davidson, Davis, Green, and Fellows, 1963). The conclusions themselves, if warranted, indicate the importance of RNA in brain functioning but may not indicate the mechanisms involved. They may not distinguish RNA as the

specific bearer of information unique to the memory process from RNA which is a normal and necessary intermediate in all cellular function. Furthermore, they may not enable us to differentiate a molecular theory of memory from a cellular one.

When conditioned planarians were transected, regeneration of the tails in the presence of ribonuclease presumably altered the retention level of the specific task (Corning, and John/1961). The heads regenerating in ribonuclease displayed a retention level equal to that of head and tail sections which had regenerated in pond water. There is no evidence offered whether RNA or any other macromolecule was ever modified by the impure ribonuclease preparations used at pond water hydrogen ion concentration or whether there were any direct or indirect biochemical effects upon neural tissue and its macromolecular components. Certainly further experiments will be necessary before any conclusions can be drawn from these data.

If RNA plays a role in memory processes, then interference with its metabolism may well interfere with the "learning and memory" process. Nucleic acid antimetabolities have been used to support the role of RNA in these processes. Dingman and Sporn (1961) demonstrated that 8-azaguanine in rats diminished correct maneuverability of a new swimming maze, although it had no effect upon performance in a previously learned maze. Brain RNA was isolated and was shown to contain 8-azaguanine-C_{14}. However, it is unclear to what extent normal RNA production and function was altered by the presence of the 8-azaguanine and to what extent the demonstrated effect upon learning was the result of the new 8-azaguanine-containing RNA species, the lack of normal RNA associated with the learning experience, or some other less direct effect upon cellular metabolism. Gerard, et al. (1963) were unable to confirm an effect of 8-azaguanine in rats running a Hebb-Williams maze, although they were able to prolong the latency time for

"fixation" of an assymetric limb-posturing by administration of 8-azagua-nine. In these experiments there are no data to indicate whether the anti-metabolite had been incorporated into RNA or whether it had produced any biochemical effect. Consequently we can not even reasonably speculate on the sensitivity of different behavioral responses to the antimetabolite.

Barondes and Jarvik (in press) were able to inhibit mouse brain RNA synthesis 70-85% by intracerebral injection of 40γ of actinomycin D, but were unable to affect either the acquisition or retention of a "learned" response. Control mice were placed in a cage where they would be shocked for any activity by making contact with metal plates on the cage floor. After several shocks their activity was sharply curtailed, and the animals would remain in one area. When actinomycin was administered the behavior was not significantly altered and the experiment was considered negative. One of the problems is that if actinomycin significantly diminished an animal's activity in the presence of shock, any behavioral effect would have been masked by the experimental design. Under these circumstances one could not have determined whether the diminution of activity was due to "learning" or to a depressant effect of the drug in the presence of shock. However, the biochemical effects of the drug were clearly delineated and RNA synthesis was inhibited 70-85% depending on the dose employed. The particular advantage of this compound is that it is known to inhibit DNA directed RNA synthesis irreversibly, and therefore exhibits a direct effect upon the production of the macromolecule rather than an indirect reversible effect as seen with the antimetabolites. However, because of this irrever-sibility the drug is extremely toxic and the animals all die after several days, so that no long term experiments are possible.

The fact that actinomycin D had definite biochemical effects but had not been shown to affect behavior prompted Dr. Lincoln Clark, Dr. Richard

Wimer, Dr. John Fuller and me to examine the influence of this compound on several different modes of behavior in mice. Mice injected intracerebrally with 10-30 gamma of actinomycin D (solubilized with mannitol) were compared to mice similarly injected with mannitol alone. At 18-24 hours there was no discernible differences in motor activity either inside or outside the cage. The mice were tested at this time in a novelty activity test in which both the latency of approach and the length of time of contact with a novel object were recorded. In both the actinomycin and the mannitol groups there was a significantly shortened latency until contact with the novel object and a lengthened period of contact with it, and there was no significant difference between the two groups.

A second group of mice were similarly injected with actinomycin or mannitol and were tested on the acquisition or retention of a swimming maze. The maze was the Waller T maze with the central channel painted gray, one of the arms painted white, and the other black. The escape ladder was always located at the end of the white arm and its position as a right or left turn was altered on a random basis. For uninjected as well as injected C57/B16 mice approximately 30-40 trails were required to reach 90% criterion with an error being considered any turn away from the ladder; and this level of performance could be maintained for longer than one week. Sixteen animals injected with 20γ actinomycin D or with mannitol and tested 18 hours post-intracerebral injection made approximately the same number of errors in attempting to learn the maze. Similarly no effect of actinomycin could be demonstrated upon the retention of this swimming maze performance, either in errors made or in time necessary to swim the maze.

The 51 animals which were tested for retention of swimming maze maneuverability were then tested for their ability to jump onto a ledge from a grid floor when a shock was passed through the grid. The animals were

removed from the box if they had not escaped to the ledge after 60 seconds of shock. The mean times required for the mannitol injected animals to jump onto the ledge were 29, 9, 7, 6, and 5 seconds on five successive trials; whereas the mean times were 53, 35, 30, 32 and 34 seconds for the actinomycin injected animals on five similar trials. In this test actinomycin had significantly altered the animal's behavior. The differences in latencies in the first trial in the actinomycin and mannitol injected animals may represent differences in pain threshold or motor activity. However, the reason for the actinomycin-injected animals failure to improve their performance in the jumping box on subsequent trials at the same rate as the mannitol-injected controls is unclear. Unfortunately these preliminary observations were not extended at that time to determine what specific aspect of the behavior had been affected by the drug and whether any information storage had been interrupted. All animals tested gave evidence of their ability to jump. Furthermore, none demonstrated a generalized motor deficiency. All were rerun in the swimming maze, and both accuracy in swimming the maze and ability to traverse it in less than 10 seconds were preserved in actinomycin as well as mannitol injected animals.

Of the three types of behavior tested, performances in the novelty activity box and the swimming maze were unaffected by 20 Υ actinomycin/animal, whereas this same dose of actinomycin definitely affected escape behavior in the jumping box. These experiments have now been extended by Dr. Lincoln Clark to include an effect of actinomycin on a conditioned pole escape response in the deer mouse. After intracerebral injection of the drug, retention of avoidance but not escape behavior is altered. Several hours later retention of escape behavior also is affected, and still later generalized incoordination develops followed by tremulousness, circling movements, abnormal respiratory activity, generalized seizures, and death.

The extent of inhibition of RNA synthesis was evaluated in 10 of the tested C57/B16 animals as well as in 20 CF strain white mice which had no behavioral tests. Orotic acid $6-C_{14}$ was injected 18 hours into animals receiving 20Y of actinomycin or the corresponding mannitol inoculation. Since the incorporation into RNA appeared linear for at least 60 minutes, the brains were removed after 45 minutes, homogenized, and divided into 2 aliquots. From one aliquot, RNA was isolated by the hot phenol technique of Scherrer and Darnell (1962); and from the other it was characterized by alkaline hydrolysis of an acid precipitate according to a modification of the technique of Schmidt and Tannhauser (Fleck and Munro, 1962). Both techniques gave values of 60-70% inhibition of RNA synthesis in the actinomycin injected animals compared to the mannitol injected ones. Furthermore, the degree of inhibition was the same in both strains of mice.

Having demonstrated an alteration of behavior by a drug which inhibits RNA synthesis, we still can make no definitive statement about the role of RNA in central nervous system functioning. We have demonstrated an alteration of RNA associated with an alteration in an animal's response, but we do not understand what function the alteration in RNA serves. RNA is present in all cells; and its synthesis and metabolism are important in cell function. Interference with normal RNA synthesis would then be expected to interfere with cellular metabolism; and one cannot separate injury to RNA as the seat of the memory trace from injury to RNA as a vital constituent in intracellular metabolism which will affect intercellular communication and the input, storage, or readout stages of the memory process. In brief, there seems no way to the present author to distinguish a sick molecule from a sick cell or group of cells in all the previously described experiments including our own. Furthermore, we have not excluded the possibility that all these experiments have been merely chemical ablation studies where

we have affected certain groups of cells which can concentrate the antimeta-
bolite and inhibitor or are especially sensitive to it. Such an interpre-
tation would be suggested by the fact that we were able to alter behavior
when only 60-70% of RNA synthesis was inhibited. This would also be sug-
gested by the experiments of Flexner et al. (1963) where interruption of
avoidance behavior was associated with presumed inhibition of protein syn-
thesis by puromycin; and the site of the drug injection was of paramount
importance.

At this point we shall turn to the experiments of Hyden to determine
whether the analysis of a single cell can obviate some of the difficulties
mentioned. Hyden (1962) studied the base composition of nuclear RNA in
Deiter's cell of rats that learned to balance on a wire to reach food on
an elevated platform. This was compared to rats fed ad. lib. who were
passively rotated on a revolving platform. The whole cell from both groups
compared to a third unstimulated control showed an elevation of total RNA
as measured by the photographic-photometric technique. Twenty-five nuclei
were pooled, extracted with phenol followed by ribonuclease and analyzed
for individual bases by the microelectrophoretic technique of Edstrom
(1960). With this technique total nuclear RNA did not increase but its
base composition was found to be altered in 4 animals in the "learning"
situation; adenine was increased and uracil decreased. This shift in base
composition was interpreted as the synthesis of a new RNA in response to
the "learning" situation. However, no direct demonstration could be offer-
ed of the synthesis of an RNA unique to the particular situation, nor could
any experimental evidence be offered to determine whether the total cellu-
lar RNA increase reflected increased levels of ribosomal RNA, soluble RNA,
or messenger RNA. The technique itself may not exclude an increase in small
molecular weight RNA fragments or in nucleotides that merely represent an

expansion of the pools within the Deiter's cells. Nor can we be certain that analyzing the products of ribonuclease disgestion under the given conditions gives an accurate picture of the total macromolecular RNA constituents present.

The most important question raised by these experiments is whether stimulation of a cell per se is sufficient for memory storage, or whether as Hyden implies, the learning process may involve the cell in a qualitatively rather than quantitatively different fashion. Hyden compared his "actively" stimulated experimental group to "control" rats whose Deiter's cell was presumably stimulated to the same extent by "passive" means; but this assumption was not confirmed by direct electrophysiological measurements. It is therefore possible that the two situations of vestibular stimulation may not represent a learning and a non-learning situation as much as differences in the extent and source of activation of Deiter's cells. In the "control" animals, the passive rotation on a disc may result in less interaction of brain stem vestibular nuclei with the remaining neuraxis than the range of interaction of the motor and sensory systems which would be implicated when a rat has to ascend a wire to a 75 cm height in search of food. The experimental situation would of necessity require the coordination of large numbers of cells throughout the neuraxis and musculature. The actual data may, in fact, support this interpretation because RNA in Deiter's cells from "passively" stimulated animals increased less (from 683 to 722 uug RNA/cell) than it did in the "actively" stimulated learning situation where the increase was from 683 to 751 uug RNA/cell. The single cell may not ever know that it is involved in a learning situation or not. As a neuron, it represents a cell highly differentiated for intercellular communication whose response may differ with different levels of stimulation or perhaps with different patterns of stimulation. It seems reasonable that both the

extent and the pattern of stimulation would be different when Deiter's nucleus is receiving new information from the peripheral vestibular apparatus compared to its receiving information simultaneously from peripheral, central, and visceral sources. If the base composition changes are real, what seems most significant about Hyden's experiments is not that he may have chemically characterized the participation of Deiter's cells in a learning versus a non-learning situation, but that he may well have characterized a difference in intracellular response to two different levels or patterns of stimulation.

Indeed, it is the effect of different levels of patterns of activity upon neuronal metabolism which may improve our understanding of neuronal function. But can this define the memory process? Many experiments have indicated the striking alteration in nervous tissue constituents, including RNA, with increased or decreased stimulation (Brattgard, 1952; Geiger, Yamasoki, and Lyons, 1956; Horowicz and Larrabee, 1962). Yet we still cannot define the way in which these alterations may be significant. Even understanding metabolic events in a single neuron will only be significant if we can delineate the manner in which these metabolic events will affect the neuron's participation in a communal response. It is the communal response of groups of neurons organized in pathways and interacting with each other in complex networks which characterizes the overall functional categories of a memory system. In such a scheme new information would be defined as an altered pattern of communication between at least two cells whose previous communication had been either inefficient or non-existent. Establishment of the memory trace would be associated with improved communication between cells; storage of the memory with the maintenance of the new communication pattern; and recall with the sequential activation of cells participating in the pathway determining the particular response. If we are to

characterize the role of any single cell involved in the storage of this new
information we must specify not only that a given biochemical parameter has
changed as a result of prior activity but also that the new biochemical
state directly affects the pattern of intercellular communication. Whether
any altered biochemical state can affect intercellular communication will,
of course, depend upon whether the relationships between any two cells may
be modified by previous activity.

The point of approximation of two communicating cells, the synapse,
has often been implicated as the site where transient or permanent changes
may influence information transfer (Ramon y Cajal, 1910; Hebb, 1949). The
body of the axon itself conducts in an all-or-none manner and probably is
incapable of significant modification, but most other points in the cell
contribute to impulse transmission with graded responses and are therefore
potentially modifiable. Unfortunately, the experimental data on the basic
mechanisms of intercellular communication within the brain are limited; yet
there are sufficient data from neurophysiological studies on the neuromus-
cular junction and spinal cord to permit reasonable speculations about cen-
tral processes. When a wave of depolarization passes down an axon into its
terminals, neurotransmitter is released (Fatt, 1954). At the neuromuscular
junction, this transmitter is acetylcholine. It ordinarily is bound, and
is released in packets even in the absence of axonal impulses. Apparently
the function of the impulse is to increase the frequency of release of these
parcels, but it normally has no effect on the size of the packets released
(del Castillo, 1954). The neurotransmitter reacts with receptors in the
post-synaptic membrane, resulting in an alteration of membrane polarization
and intracellular ionic constituents. When polarizations of the synapses
are of sufficient size to depolarize the initial segment of the axon, an
all-or-none action potential will be generated. The amount of acetylcholine

per packet is probably regulated by its rate of synthesis; whereas the rate of acetylycholine released from the presynaptic cell will be determined by characteristics of the axon terminals themselves as well as by the activity of other axons which may impinge on these terminals (e.g. presynaptic inhibition (Eccles, 1956)). Therefore, altering the amount or the frequency of transmitter release would definitely modify communication between the involved cells. Furthermore, the size of the recorded potential in the post-synaptic cell will depend not only on the magnitude of acetylcholine released but on, 1. the local activity of the cholinesterase which hydrolyses the acetylcholine, 2. the sensitivity and density of the receptors which combine with acetylcholine to cause an alteration in membrane permeability, 3. the resistance and potential of the post-synaptic cell membrane, and 4. the electrical and biochemical characteristics of the neuronal cytoplasm which may affect transmission of the local polarizations at the synapse to the initial segment of the axon where the all-or-none impulse will be initiated. Alterations of the post-synaptic membranes, the receptors or enzymes which inactivate the transmitter, or the post-synaptic cytoplasmic constituents or complex dendritic arborization will have definite effects on the conversion of input into output. For example, a synapse associated with a large dendrite with extensive surface area and high cytoplasmic ionic strength will facilitate passage of the active state to the initial segment of the axon; whereas small surface area and low ionic strength might mitigate against efficient intracellular information transfer.

In essence, any chemical changes which would affect the synthesis or release of neurotransmitter, its affinity for the post-synaptic receptor, its rate of destruction, the post-synaptic membrane properties or the nature of the post-synaptic cytoplasm might alter intercellular communication and information transfer. The maintenance of these biochemical changes and of

the new pattern or level of intercellular communication need not depend upon the indestructibility of any single macromolecule. What would be required instead is that the new steady state of these biochemical alterations be maintained. Activity could induce transient biochemical effects which may in turn facilitate subsequent activity by modifying one of the aforementioned points in impulse transmission. This new activity would in turn reinforce the biochemical changes which initially produced it. The altered pattern of intercellular communication would not then reside in any permanent macromolecular engram but in the dynamic interaction of a post-synaptic cell with the pattern of pre-synaptic influences. The effect would persist as long as the duration of the effective biochemical changes were greater than the length of time between an initial and subsequent activations of the pathways involved.

The difficulty with implicating increases or decreases of cellular RNA in information storage is that we doubt that this macromolecule plays a direct role in intercellular communication. If it plays any role at all in information storage processes, it is probably as a template for synthesizing protein constituents which directly affect cell interactions. The important biochemical changes that we must then characterize will not be the alterations in RNA, but the alterations in the proteins whose presence may directly affect intercellular communication. Assuming that RNA is required to synthesize a protein which would modify impulse transmission, the half-life of the protein and not the half-life of the "messenger" RNA would be critical in maintaining the new state. Presynaptic transmitter may well increase RNA production and in turn protein production in the same way that hydrocortisone may increase de novo protein synthesis in the liver through DNA activation and messenger RNA synthesis (Garren and Howell, 1963). However, no molecule alone would be sufficient to specify the permanent storage of information

when such storage is defined as the maintenance of an altered communication between at least two cells. The presynaptic transmitter, interacting with the post-synaptic receptor, and directly or indirectly affecting post-synaptic cytoplasmic constituents might represent one half of an intercellular feed-back mechanism. The other half might be determined by the effect of the altered intracellular biochemical state upon the receipt or transmission of future information. No single component could be omitted for effective operation; and the engram could only be defined by both the effect of activity on the biochemical state and the effect of the biochemical state on subsequent activity.

From the available neurophysiological literature, what appears most striking is that under normal circumstances neurons may not be functioning at maximal efficiency in converting presynaptic impulses into post-synaptic ones. Within the spinal cord, for example, passage of an afferent impulse at one time may result in no response within the post-synaptic cell; whereas at another time similar presynaptic stimulation will cause a post-synaptic response (Kuno, 1963). If the nature of the interaction were modified so that each presynaptic axonal impulse elicited a post-synaptic response, this would represent a significant alteration in the communication pattern. Post-tetanic potentiation is another example of altering information transfer between cells. Following single or repetitive presynaptic impulses there is often potentiation of the post-synaptic response that is elicited by a test impulse in the same presynaptic fibre (Eccles, 1957). This has been attributed to an increased output of transmitter substance in the presynaptic terminals (Eccles, 1957). Unfortunately, no convincing physiological experiments have demonstrated a change in response of several cells which will significantly outlast the period of activation. However, it must also be pointed out that cellular recording is not usually sampled over

periods of days and long term neuronal plasticity and modifiability can be neither confirmed nor denied. Changing the number of synapses also represents a reasonable means of changing the pattern of cell communication and the process of information storage. However, as long as the cell is not normally functioning at peak efficiency with the machinery it already possesses, it seems reasonable to speculate that an intercellular relationship might be modified by improving its present machinery rather than by adding more suboptimal units.

In reviewing the experiments which purport to demonstrate a central role for RNA in learning and memory, we have found that they are insufficiently conclusive to implicate RNA at any stage in the learning process. Where RNA can be implicated on the basis of biochemical data, the specificity of its participation in the memory process must be questioned; and we must necessarily ask whether the effect was determined by a sick molecule throughout the brain, or groups of strategically located sick cells. We must recognize that in a study of mammalian neural function we are dealing with an infinitely complex system which has become specialized for intercellular communication and which contrasts with individual bacteria responding as isolated units to the environment. What appears most important for the nervous system is to characterize these cellular interactions biochemically; namely, to determine how the spatial and temporal summation of synaptic events on a cell will affect a cell's membrane, nuclear, and cytoplasmic constituents and how these changes will alter both its own subsequent excitability and its ability to communicate these changes to another cell by altered events at its own axon.

The role of RNA may be significant, although only as a link in a chain of intracellular events. As a result of cellular activity, newly-synthesized RNA may translate the genetic potential of the cell into specific

proteins with defined effects on impulse transmission. However, the RNA itself would not be the storage engram. Storage would, in fact, be defined by an increased steady state level of biochemical constituents including RNA which would be maintained by random or specific activity, and which would, in turn, improve the normal sub-optimal efficiency of cell communication. Neurophysiologists are presently attempting an electrical characterization and conformation of these points. We should initiate a similar biochemical characterization.

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NUCLEIC ACIDS AND BRAIN FUNCTION

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Recent work with the macromolecules involved in the transfer of genetic information in protein synthesis has been outstanding. Such work suggests the possibility that these same macromolecules (deoxyribonucleic acid-DNA, ribonucleic acid-RNA) may play important roles in the functioning of the brain for complex events such as learning.

A number of investigators have conjectured that RNA is the basic molecule involved in learning events and have reported results which are not inconsistent with the conjecture (Beckwith, 1962; Cameron and Solyom, 1961; Cook, et. al., 1963; Corning and John, 1961; Dingman and Sporn, 1961; Gerard, et. al., 1963; Hyden, 1959, 1961; Zelman, et. al., 1963). Others have suggested that DNA or DNA complexing might be the basic mechanism (Dingman and Sporn, 1963; Gaito, 1961, 1963). However, the research so far has not been able to clarify the role of nucleic acids in the brain's control of organized behavior during learning; there is no conclusive evidence to indicate that either of the nucleic acids is <u>directly</u> involved in learning.

There are a number of different events within learning which any biological approach must handle. These include short term or transient memory, long term or relatively permanent memory, reactivation of long term memory traces, normal forgetting, and unusual forgetting (such as in hypnosis, amnesia, and psychological repression). The most important event of all is the mechanism by which the contributions of the various portions of the brain are coordinated and integrated into organized behavior. How can the nucleic acids perform these functions? Although some individuals (e. g., Hyden, 1959) have attempted to build a theory around the nucleic acids, the attempts

so far have not been overly satisfying.

Even though the research results are inconclusive concerning whether DNA or RNA, or either, are basic for learning phenomena, one result is clear-cut enough to be useful in attempting to determine brain function during complex events. During sensory stimulation and learning events, amounts of RNA increase rapidly and then decrease within minutes. For example, Geiger, et. al. (1956) stimulated the cerebral cortex of cats and found a change of nucleic acids in the stimulated areas, which was reversible in minutes. Hyden (1961) has reported similar results.

Related to this rapid increase of RNA is the fact that several types of DNA have been reported. Swift (1962) concluded from his studies on DNA in species of flies that there are two types of DNA: one which is constant from cell to cell and another varying in various cell types at particular stages of ontogeny. Likewise, Bendich, Russell, and Brown (1953) found two types of DNA in growing rat tissue, one showing greater amounts of turnover than the other.

Recently, Sampson, et. al. (1963) have reported two DNA fractions in plants: a high molecular weight stable fraction and a low molecular weight fraction showing a relatively rapid rate of turnover. The proportion of the two forms varied with physiological state and with the type of tissue. Male germinal tissue contained negligible amounts of the low molecular weight form. Growing regions of root and leaf had as much as 20% of their total DNA in the low molecular weight form. Dormant embryos in wheat seeds had about 10%, but the amount increased sharply on induction of germination. The high molecular weight DNA was found to have the same composition irrespective of tissue origin, 55% of guanine-cytosine. The low molecular weight DNA in roots of both wheat and corn had greater guanine-cytosine content. Sampson, et. al. indicated that in respect to the properties investigated, the high

molecular weight stable form behaved typically for genetic material. However, the low molecular weight active DNA appeared to be performing a physiological role.

A phenomenon involving active DNA is enzyme induction. It has been indicated that the presence of certain chemicals within an organism can lead to changes which allow the cells to produce more of a specific enzyme on a later occasion. According to the model proposed to account for this event (Platt, 1962; Sypherd and Strauss, 1963), the DNA of structural genes are activated or deactivated by regulator and operator genes. Hormones can play a role in this induction process; Kenney and Kull (1963) have found that hydrocortisone increases the rate of synthesis of liver nuclear RNA during the induction of tyrosine transaminase in adrenalectomized rats.

Investigations of chromosomes of insects have shown puffing at specific chromosomal sites which are assumed to be sites of activity. These sites of activity appear to be at different loci in each tissue and at different loci in the same tissue at various times. Karlson (1962) has reported that a hormone, ecdysone, is able to bring about chromosomal "puffing" in the insect, Chironomus, and soon thereafter the organism goes from the larva to the pupa state. During the "puffing," rapid RNA and protein synthesis occurs in the puffed region (Karlson, 1962; Schneiderman and Gilbert, 1964).

Bonner has suggested that proteins complexed with DNA regulates DNA activity. Huang and Bonner (1962) found with pea embryo chromatin that when the protein fraction, histone, was removed, the rate of RNA synthesis increased fivefold. Further work (Bonner and Huang, 1962a) showed that the chromatin contained 80 per cent DNA bound to histone and 20 per cent free DNA. They suggested that the function of histone was to bind DNA and block the transfer of "information" from DNA. Bonner and Huang (1962b) also discussed lack of inhibition following a reduction of histones in certain plants

in transition from the vegetative to the flowering state. Accompanying this reduction was a dramatic increase in RNA concentration in the cells. Likewise, Izawa, Allfrey, and Mirsky (1963), working with the giant lampbrush chromosomes of amphibian oocytes, found that the addition of thymus gland histones to isolated nuclei inhibited RNA synthesis.

In some of the studies concerned with active DNA (e.g., Izawa, Allfrey, and Mirsky, 1963), it has been indicated that the relative amount of RNA per unit DNA (i.e., the $\frac{RNA}{DNA}$ ratio) is greater for active than for non-active DNA sites. Such results would suggest the possibility of identifying the brain loci for short term memory by sacrificing animals while they are learning and locating sites of rapid RNA synthesis using the $\frac{RNA}{DNA}$ ratio. The mechanisms underlying short term memory may be more drastic and easier to detect than those for long term memory. Thus the $\frac{RNA}{DNA}$ ratio would provide an index for brain function. By a number of experiments one might determine crucial loci contributing during learning events.

Research is underway making use of this result of rapid RNA synthesis. There are three stages to the experimentation, in each we are concerned with answering a single question.

Stage 1. What brain loci show rapid RNA synthesis during learning events?

We are attempting to use the RNA/DNA ratio as an index of brain function, thus allowing for a mapping of brain areas relative to behavioral events. At present we are doing some preliminary experiments in which animals undergo about 10 to 15 minutes in an operant conditioning apparatus learning that pressing of a bar will deliver food. During learning the animals are sacrificed and liver, kidney, and brain sections are dissected. The brain is sectioned into ten parts--anterior, medial, and posterior portions of the dorsal cortex, the same portions of the ventral cortex, cerebral hemispheres

less the cortex, cerebellum, upper brain stem, and lower brain stem. Non-learning control animals are sacrificed and the same tissues removed. We then extract DNA, RNA, and proteins from each portion with a modified Schmidt-Thannheuser procedure and compare the RNA/DNA ratios of control and experimental animals. The basic assumption is that during learning or other behavioral events some DNA sites will be activated or derepressed so that rapid bursts of RNA synthesis will occur.

In these first experiments we are confounding learning with sensory stimulation and motor activity and will later control for these; however, at this time we are merely concerned with whether the RNA/DNA ratio will be able to discriminate between groups of animals in grossly different conditions. We have been proceeding slowly and have run only a few animals but some results seem relatively clearcut. The anterior portion of the dorsal cortex in the rat (according to electrophysiological criteria, the motor area) has shown a much greater RNA/DNA ratio in experimental than in control animals in several cases. This may be merely indicating the effects of motor activity, however. One portion of the brain, the posterior ventral cortex, shows results which suggest that it may contribute significantly to the visual motor learning task. On the other hand, in no case has the ratio been different in the cerebellum. Several other tissues also appear not to contribute significantly in the learning task, i.e., cerebral hemispheres less the cortex, liver, and kidney.

One problem with the above approach is that the increase in RNA during various behavioral events may be so small compared to the amount of RNA normally in the cell that the RNA/DNA ratio will not be discriminative, i.e., the "noise" level will be too great. Thus to improve our present approach we plan to extract only nuclear DNA and RNA. This procedure will eliminate the cytoplasmic RNA which constitutes most of the RNA in the cell and allow

the detection of RNA increases as they are occurring.

Stage 2. Are the increased amounts of RNA incidental or necessary for learning?

This question will be handled by injecting rats with actinomycin D (an inhibitor of RNA synthesis) and the effects on the amount of RNA in critical loci and on learning ability will be noted. If RNA is necessary for learning, there should be a reduction of RNA in the critical loci and an impairment of learning ability. If RNA synthesis is depressed but learning ability is not affected, the RNA synthesis would be considered to be incidental in learning.

Stage 3. Is the RNA synthesized during learning events a new species?

If the RNA is shown to be necessary for learning, biologically active DNA and RNA will be extracted from critical brain loci, non-critical brain loci, and from non-neural tissue (e.g., liver). The DNA-RNA hybridization experiments will be performed. If there occurs a lesser degree of hybridization between DNA from the liver and RNA from critical brain loci than between DNA and RNA from the liver or between DNA from the liver and RNA from non-critical brain loci, this result would suggest that learning involved a new species of RNA. If the degree of hybridizing was the same in all three cases, this would suggest that a change in DNA regulating mechanisms allowed increased amounts of RNA to be synthesized during learning events. Thus one would be interested in the investigation of DNA and DNA complexes with other molecules, e.g., as in fractionation of active and stable forms of DNA as suggested above under Stage 1.

This three stage investigation on short term transient memory events should provide information of value for research on long term (relatively permanent) memory, reactivation, forgetting, and overall integration, problems which are so little understood at the present time. The results of the

preliminary work are encouraging and have suggested the possibility that interesting and basic results may be imminent.

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RNA AND MEMORY*

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Friends, Kansans and Biochemists--lend me your fears. Some of you have come to bury RNA; I come to appraise it. And whilest yet you wrap the grave-yard shrouds about it somewhat hurriedly, I announce to all who'll listen that the rumors of its death are greatly exaggerated.

The problem we face, as Dingman and Sporn have put it so succinctly, is this: What evidence do we have (if any) that RNA, or any other single mole-cule, acts as the sole engram of a memory trace? After reviewing the litera-ture pertinent to the molecular theories of memory in their recent Science article, Dingman and Sporn draw several conclusions. First, they state that the results of a large number of experiments strongly suggest that RNA is intimately linked to memory storage. With this conclusion, I heartily con-cur. Next, they state that no data yet reported prove that the engram is RNA and only RNA. Obviously, this statement is correct, or our present con-ference would have rather a different title, purpose and scope. Their final point, however, is the most interesting and one deserving of further com-ment. They write as follows: "A comprehensive theory of the structural basis of memory must also consider the function of the entire neuron, with consequent emphasis on the reciprocal relationships between the cell body and the synapse, as well as the complex functional interrelationships between neurons." Now, this conclusion is an honorable one--as Dingman and Sporn are honorable men. Indeed, their point is so honorable and virtuous that to ex-pect a mere psychologist to disagree with it is akin to expecting a politician

to make a public attack on the virtue of virtue. Let me show you, though, how in saying so much, they have managed to say almost nothing at all new.

Let us suppose that a week from now, some unknown scientist publishes the definite experiment showing that the engram is encoded by means of a structural change in RNA and only RNA. You will admit that such an hypothetical finding would badly damage Dingman and Sporn's present position--yet their final point, virtuous as it is, would remain as valid as ever. Obviously RNA must exist in a cell, obviously one must consider the functioning of the entire neuron and the complex relations which exist among the various parts of the nervous system. William James said much the same thing, with diagrams, 70 years ago. But what does this have to do with the engram? One might as well state that, since the liver is necessary for life, and life necessary for learning, the liver is intimately associated with learning and hence part of the engram. Indeed, while considering the Dingman and Sporn position, I was reminded of that interesting pedagogical theory which insists that one must "teach the whole child." Now, the only proper response to the latter-named educational theory is this: "Yes, but don't you want to teach the whole child something," and I suspect that the only proper reaction to Dingman and Sporn's present position on the "complex functional interrelationships between neurons" is this: "Complexities are fun but RNA ain't undone."

A year ago, at a symposium held at the Midwestern Psychological Association meetings in Chicago, I came forth with what I called my "tape recorder theory of memory." It wasn't really a theory, it was more a statement of faith, and the best thing that can be said of it is this--it was so vaguely phrased that it could not possibly be disproved. At the end of that speech, I mentioned some research involving RNA and rat brains that I had hoped to undertake, I leapt to a conclusion, I drew a moral and I told a dirty joke.

The joke you've all heard before, so I won't bother to repeat it. The conclusion to which I came was a simple one--if RNA is indeed the memory molecule, we might well be able to alleviate many human problems (such as addiction and some types of mental retardation) by using RNA in a therapeutic manner. Given the premise that RNA is indeed the engrammatic substance, my conclusion would follow and in trevial.

The moral which I drew was this: "Interdisciplinary research must be interdisciplinary if it is to be good research. The field of molecular biology at the moment is crowded with chemists many of whom know practically nothing about the science of behavior, with psychologists who know nothing about chemistry, and with a few physiologists whom I suspect aren't very good at either." Very little that has appeared in print during the last year suggests that my moralizing had much effect. I am sure that I know as little about chemistry now as I did then, though I am struggling to learn, and I remain convinced that most of the non-psychologists in this field are as baffled now by behavioral variables and psychological jargon as they ever were. Let me illustrate this final point. In their Science article, Dingman and Sporn write as follows: "Studies on planarians have indicated that ribonuclease blocks the retention of a conditioned response in regenerating planarian tails, and it has been claimed that learning is transferable from one planarian to another by way of cannabalistic ingestion. However, the interpretation of the cannibalism data is by no means straightforward, since it appears in these experiments it was transfer of the general capacity to learn, rather than transfer of the specific learning of a particular task."

Now, it is the latter part of this quotation which bothers me most. The only reference cited by Dingman and Sporn which is pertinent to the cannibalism studies is my own 1962 article which appeared in the Journal of Neuropsychiatry. Let me assure you that nothing reported in that 1962 paper

could give rise to an interpretation that it was the transfer of "the general capacity to learn" that we achieved in our planarians. You see, most psychologists believe that "the general capacity to learn" is genetically determined, and I don't believe that our results were all that genetic. I would imagine that what Dingman and Sporn really meant was this--whenever an animal, such as a rat, is first placed in a training situation such as a maze, it learns not only the correct pathway to the goal but also a wide variety of responses which are not necessarily directly associated with the goal pathway itself. These latter-named, secondary responses have to do with the animal's becoming accustomed to being handled, to eating in the maze, to not being frightened by its new environment, etc. It may also learn that it has to learn and, beyond this, it may after acquire what Harlow has called a learning set. As we all know, rats allowed to explore a maze for some time prior to being trained in the apparatus show much faster learning than do rats (or worms, for that matter) which are not given this prior experience. It is likewise true that under many circumstances, rats and worms learn a second task more rapidly than they do a first task of equal difficulty.

The important points about all of this are the following: First, an animal in a training situation doesn't show just "the specific learning of a particular task," but rather demonstrates the specific learning of dozens, even hundreds, of particular, secondary tasks associated with the training situation. In our experiments we may measure only the performance of the primary task, but the learning itself is multiple; and it is the build-up of hundreds of very specific habits which accounts for the change in the animal's behavior we call "learning." Thus, even if Dingman and Sporn have unpublished data which suggest that it is such secondary learning which is being transferred in planarians, it is still the case that engrams, and not "general capacities," are being cannibalized.

It is also true, of course, that even if it were "general capacities to learn" which got passed along rather than "specific learning," we'd still face the problem of what the transfer mechanism was and how these general capacities became functional when ingested by a planarian. Somehow, I just can't quite see my cannibals devouring "complex functional interrelationships between neurons."

Now, I did not launch into this lengthy harangue to prove that Dingman and Sporn, being chemists, should refrain from hypothesizing about psychological variables; rather, I wished to illustrate one of the pitfalls which lie ready to entrap such hypothesizers. But there is yet a greater danger lurking in the scientific woods, always lying in wait to ensnare the unwary theorist. This is the spectre of the "new discovery," which could be published tomorrow, which might well knock the foundations out from under any pinnacle of theory. With your permission, I would like to cite some new evidence which just may be published in the next few months and which, I think, suggests that this conference won't turn out to be a wake held in honor of RNA after all.

In that speech I made a year ago, I mentioned that my associates and I hoped to train rats, extract RNA from their brains, and inject this into the brains of untrained animals to see if we could achieve a transfer of some kind; we planned to do the same sort of study using planarians, of course. To date, we've actually performed neither experiment. Our preliminary investigations of the effects of environmental stimulation on the amount of RNA and DNA present in rat brains were quite negative, so, unfortunately for us, we decided to postpone work on this study for a while. We had frankly hoped to be able to do the RNA extraction study in planarians much more easily, but it has taken almost the entire year for the biochemists with whom we work to develop an effective means of extracting un-degraded RNA from whole planarian

bodies with reasonable yields and purity. Hence, the first injection study
is only now underway in our laboratory -- the results won't be known until
some time this summer, and then only if all goes well, of course. Meanwhile,
we have been scooped by two other labs, and it is their findings I'd like to
report to you right now.

As a preface to the first report, let me say that I am happy to admit
that I have been greatly influenced by Hyden's thinking, for it was he, I
believe, who first explicitly postulated that RNA might be the memory mole-
cule. In spite of the rapidly accumulating evidence that RNA was intimately
involved with the formation of engrams, later theorists began to wonder aloud
if other parts of the cell, perhaps proteins, perhaps lipids, perhaps even
DNA, might not also play an integral role in the formation of memories. Ac-
cording to these theories, even if we performed the RNA injection study, we'd
have proved precious little, since the same results might also be obtained
with the injection of other cellular components, particularly with DNA.
Well, I have a letter which I received just last week from John Rieke and
Lee Shannon, two students at Pacific Lutheran University, which bears on this
topic. I would like to quote from their letter and then let you form your
own conclusions about their research:

"For a couple of months now we have been engaged in some planaria re-
search ourselves. Our project is being done in conjunction with a class in
experimental psychology here at P.L.U. We became interested in this area
from reading your own Worm Runner's Digest. The general idea for our partic-
ular experiment was suggested by the Corning and John experiment appearing
in the October 27, 1961 issue of Science. Their work concerned the effects
of RNAse on learning retention.

Our particular experiment has been centered around the hypothesis that
DNA is the so-called "memory molecule." Our attempts to either substantiate

or discredit this hypothesis have involved the method of enzyme digestion using DNAse. Using light-shock classical conditioning we have trained difr ferent groups of planaria. We have repeated the previously done cannibalism experiments in order to prove to ourselves that learning could be transferred via cannibalism. We have trained a couple of groups of planaria, minced them quite finely, soaked them in a buffer solution of DNAse, and then fed those soaked pieces to naive planaria. Each time these naive planaria have shown a complete and immediate acquisition of learning, just as did the above mentioned cannibals.

It would seem that our hypothesis is disproved, but this is not yet the case. We have sent a sample of our enzyme-soaked planaria pieces to Dr. William O. Rieke at the University of Washington School of Medicine. Using a type of paper chromatography and spectrograph he has informed us that we have not succeeded in breaking down all of the DNA molecules in our minced pieces. There are several possible reasons why this is so. We are now trying to improve our method of enzyme digestion."

You will note that, while they haven't been able totally to destroy the DNA in their minced pieces of planarians, they obviously succeeded in destroying a great amount of the DNA present. Yet, in spite of this destruction, they found that their cannibals showed "complete and immediate acquisition of learning" even when fed on DNA-impoverished tissue. I cannot tell you at this time how well-controlled their study was, for this is my first and only communication with these gentlemen. I am encouraged, however, that their findings were directly opposite to their prior hypotheses, a result which indicates their work might not suffer excessively from "experimenter bias."

If the Rieke and Shannon study can be replicated (and, let me warn you, we intend to make the attempt immediately), we can scratch DNA from the list

of transfer agents. Which leaves us with proteins, lipids, and, of course, with RNA. Let me now give you the results of another study, results so important that perhaps our present conference should have begun rather than ended with their announcement. I have reference to a series of studies performed by David J. Albert, who is presently finishing his doctorate with D. O. Hebb at McGill. Forgive me if I describe not only the experiments themselves, but also their historical background, in some detail. And I should tell you ahead of time that I quote, for the most part, from an informal summary of his work which Albert was kind enough to send to me recently.

Jan Bures, the noted Czechoslovakian physiologist, has shown that the rat needs but one functional cortical hemisphere to learn a task. If one hemisphere of the animal's brain is irrigated with potassium chloride, a slow wave of depression spreads across the affected hemisphere making it non-functional. Yet, if one side of the cortex is depressed just before the animal is trained, the rat will still show learning although the memory will be retained only on the non-depressed part of the cortex. In short, learning does not transfer spontaneously from the trained to the untrained hemisphere even when the depression is lifted. Albert made use of the spreading depression technique in an attempt to achieve a chemical, rather than a so-called "functional," transfer of learning from one side of the brain to another. Let me now quote him directly:

Procedure for Obtaining One-Trial Interhemispheric Transfer

Two groups of animals are taught an avoidance response on day one with side A of the cortex depressed; learning, therefore, occurs only on side B. On the following day one group, the Transfer Group, is given a single trial with both hemispheres functional (transfer trial); the learning on side B should transfer to side A on this trial. Another group, the No-Transfer Group, is given a single trial with side A again depressed; spreading depres-

sion on side A should prevent the learning on side B from transferring to side A. A third group, with no previous training, (the Transfer Trial Control Group) is given only a single trial with both hemispheres functional; this group controls for the learning that occurs in the Transfer Group during the transfer trial.

The following day each group is tested with side B depressed so that performance is now dependent on the previously untrained hemisphere. In the No Transfer Group and in the Transfer Trial Control Group the animals require as many trials to learn the avoidance response on hemisphere A as they had on hemisphere B. Without a transfer trial, therefore, the learning remains unilateral and does not transfer between test periods. With a transfer trial, the Transfer Group shows highly significant savings using hemisphere A.

The Transfer Group illustrates the basic preparation used in the following experiments to study the consolidation of the transferred learning. Following transfer, cortical spreading depression or cortical polarization is used to interfere with the consolidation of the learning on the hemisphere receiving the learning, side A. The animal is then tested for retention of the learning on that side the following day.

The Effect of Spreading Depression on the Consolidation of Learning

Bures has shown that cortical spreading depression interferes with consolidation and the present experiments have verified this. It is also desirable to know whether other structures not directly affected by cortical spreading are also involved in consolidation. Several structures were examined by disturbing their activity following transfer: amygdala (procaine microinjection), midline thalamus (procaine microinjection), and hippocampus (spreading depression). No effect on retention was found by unilaterally disturbing these structures following learning. It is, therefore, tentatively concluded that cortical processes are primarily involved in the long period of consoli-

dation that is found for this kind of learning.

Further experiments with cortical spreading depression show that both the time after learning that spreading depression begins, and the length of time for which it remains, affect retention. The duration effect implies that spreading depression does more than simply interrupt an active process, that is, spreading depression may be cancelling some of the consolidation that has already occurred. The possibility that spreading depression does not interfere with consolidation by interupting a pattern of neural activity was examined by analyzing the effect of one or two waves of spreading depression on consolidation. The initial waves of spreading depression appear to slow rather than stop consolidation.

Since a single wave of spreading depression does not stop consolidation, it is possible that neural activity is not involved in consolidation and that neural activity of spreading depression is not what interferes with consolidation. In particular, the slow surface negative wave associated with spreading depression may mediate the disruption of consolidation. This change in the d.c. cortical potential could be conceived to have a piecemeal effect on an existing physiological state and d.c. potentials have already been implicated in the learning process by the work of Rusinov, Morrell, and others.

The Effects of Polarizing Currents on the Consolidation of Learning

Morrell and Naitoh (1962) have demonstrated that cathodal polarization of the visual cortex interferes with learning but found uncertain effects with anodal currents. Polarization of the motor cortex had no effect.

The present experiments polarize the cortex through the dura by means of an AgCl electrode through a saline bridge, current return is through the animal's back. Current strength is 13 uamps and the immediate area of polarization is 1.5 mm^2.

Cathodal current disrupts consolidation while anodal current does not. Further, a pulsating current disrupts consolidation more effectively than a constant current. Cathodal current has these effects only if the current is applied on the posterior cortex.

The mechanism by which cathodal current produces these effects is uncertain. It is likely that local changes in neural activity are produced although Morrell (1961) finds no effect of polarizing currents on the EEG of cats and rabbits. It is thought that the effects of polarizing current on consolidation are in some way related to the temporary learning produced using anodal currents (Morrell, 1961).

The Significance of the Consolidation Experiments

Both the experiments with spreading depression and polarization suggest that the most of the period of consolidation following learning does not involve neural reverberatory activity, as reasonable as this possibility is intuitively. It seems more likely that some biochemical process occurs during consolidation: a biochemical process which is not altered by neural firing under ordinary conditions but which is dependent on electrical fields existing in the brain.

The polarization experiments suggest that consolidation of learning occurs in the posterior cortex. This would imply that the engram is also stored there.

Attempts to Isolate the Engram

The experiments on transfer of information by cannibalism in planaria can be explained for the most part by assuming that macromolecules containing information are able to migrate to appropriate places in the host planarian. That is, these molecules have a selective affinity for certain cells, namely certain kinds of neurons. This might also hold for higher animals, so the present experiment was done. Using the unilateral learning preparation, the

posterior cortex of the trained hemisphere was lesioned and injected I.P.
back into the same animal. It was predicted that information-bearing mole-
cules would migrate to the homotopic area and function as stored information.

It was found that if the lesioned material is injected the animal
shows savings in learning the avoidance task when tested the following day
(during retest, the previously trained hemisphere, of course, is made non-
functional by spreading depression.) If the lesioned material is not in-
jected or if it is heated to 100° C. for 10 minutes before being injected,
there is no savings on relearning. The part of the posterior area which
must be injected to find savings is the anterior visual area.

An attempt was made to isolate the particulate fraction carrying the
active factor by means of differential centrifugation. The active factor was
found in the debris and nuclear fraction. This fraction was sonicated or
ruptured by resuspension in distilled water and recentrifuged. The active
factor was still found in the pellet.

Biochemical characterization of the active factor was carried out by
enzyme analysis and these results must be considered as preliminary: incu-
bation at 37° C. for 60 minutes with saline did not alter the active factor,
nor did incubation in trypsin (which breaks down protein). However, incuba-
tion in RNAse seemed to remove the active factor.

These studies suggest that the active factor is a large molecule, per-
haps protein or nucleic acid. It is very likely that the active factor is
associated with the nucleus, very possibly the nucleolus. The preliminary
enzyme studies suggest that the active factor is RNA.

Most of you can imagine how excited I was when I first heard of Albert's
results. If his findings are valid, and my own talks both with him and with
his associates at McGill uncovered no obvious flaws in his work, the early
theories of Hyden, Halstead, and others will have received rather a crucial

bit of confirmation. For how else, save through some mechanism of rather specific chemical coding, can one explain the fact that memory substance (presumably RNA) migrated from the site of the injection to some critical functional site in the previously untrained cortical hemisphere?

Let me close my talk by returning to Shakespeare in this, his anniversary year. Hyden, like Julius Caesar, is an ambitious man--Hyden wants to explain the facts of memory with one simple hypothesis: That RNA is the engram. At first, this viewpoint was considered quite radical (as, indeed, it still is in certain reactionary quarters). Then, for a while, it was embraced with open arms by many of us as an excellent working hypothesis. So, we went into our laboratories to test the theory. To date, and mark this well, all of our results tend to confirm the fact that RNA is the most critical substance; and there are, I believe, absolutely no studies which strongly suggest that anything other than RNA is most critical substance. It is for this reason that I think some of us have erred when we've suggested, for one reason or another, that the engram has to be something more complex than a coded RNA molecule.

I must say also that it's easy, with the clear vision of hindsight, to pick small holes in the fabric of Hyden's work, for no pioneering scientific theory can be perfectly tailored the first time it's work in public. The RNA hypothesis, for all that it may be overthrown by tomorrow's evidence, remains the best we presently have available to us, and we should not let its incompleteness, nor its apparent inconsistencies, frighten us. Which is why, at the beginning of this talk, I asked that you friends, Kansans and biochemists lend me your fears. The evil that theories do lives after them--the good is oft interred with their boners.

I believe, gentlemen, that we've pronounced the final rites over the RNA hypothesis rather a little prematurely, for it now shows intriguing signs of possessing greatly extended lifespan.

THE MOLECULAR BIOLOGY OF MEMORY - SUMMARY

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A principal problem which has emerged clearly during the course of the present Conference is that, when we consider the alterations of the neuronal network associated with learning and memory, we do not know whether we should look for alterations on the level of the making, or alteration, of synapses or on the level of alterations in neurons. An answer to this basic question is obviously essential if we wish to find out about the biochemistry of the storage of information. We have to know where to look - whether at synapses individually or at neurons as a whole.

A second and basic fact which has confronted us throughout is associated with the fact that there are different kinds of memory, four or more. Thus we distinguish the very short term memory with a half time of two hundred and seventy milliseconds, the two intermediate term transient memories with half lives of some five minutes, and the long term permanent memory. We have, in the course of the Conference, agreed that all of these memories must be basically chemical in nature. The storage of information in an electrical network requires devices with alternative states, devices which we might call flip-flops. The devices used in the short term memories are evidently ones in which the conversion from one state to the other, brought about by electrical inputs, is reversible with time. The chemical alterations associated with long term memory, on the other hand, are not reversible. We have, during the course of the Conference, discussed possible flip-flop mechanisms of a reversible nature - for example, proteins which might be converted from one physical conformation to a second by binding with a small molecule, possibly a transmitter substance. I believe that we have all agreed that it would be profitable to try to study

short term memory by looking in the cortex for proteins which might react
with transmitter substances and alter themselves specifically into differ-
ent conformations.

But the main task of our Conference has been to consider the nature of
permanent memory - of that memory which we know to be not immediately elec-
trical in nature but to be associated in some way with processes which
transform the neuronal network in such a way that information remains per-
mamently encoded in it. It is quite clear that a first and important ap-
proach to the chemical changes associated with permanent memory will be
the comparison, in a chemical way, of the cortical cells of rats either
completely sensorily deprived or completely sensorily over-privileged. A
small start along this line has been made by Hyden. More thorough studies
are, however, required. This is an important task and must be done as
soon as possible. It is basic to all other sorts of investigations on the
chemistry of memory.

It is the consensus of our Conference that increases in cortical con-
tent of the RNA and perhaps also of protein are associated with the stor-
ing of sensory information. This has been brought out in the many articles
of Holger Hyden, in the experiments of Frank Morrell, most elegantly in
the experiments of John Gaito, and others. When we think about models for
the understanding of the chemical changes associated with memory, we must,
therefore, imagine models which include the participation of alterations
in the RNA of cortical cells.

We have then quite good reason to suspect that increases in RNA content
are associated with those alterations in a neuron or neuronal network which
constitute long term memory. What further can we say about RMA and memory?
It has, of course, been suggested that information stored in memory is
stored in the form of new RNA molecules which then contain the experiential

data written out in RNA language. This thought is back of Hyden's emphasis on the fact that the RNA synthesized during learning possesses a nucleotide composition different from that of RNA made in the same cells during the course of non-learning. It is the inference too from McConnell's report that unlearned planaria can learn simply by eating their learned brothers. No thought is so fantastic that the molecular biologist should not try thinking it for a while. The present thought, however, becomes less and less appetising as one thinks about it. It requires that information encoded in electrical form be transcribed into base sequence form. It requires neurons to manufacture RNA of base sequences not specified by their DNA. This, as we all know, is very anti-party line since in normal cells of higher creatures all RNA is made by DNA-dependent RNA synthesis and the neurons of the cortex are no exception to this general rule. The thought that RNA constitutes the "memory molecule" is not in its simpler and naive sense a persuasive one. It is the belief of the present summarizer that the Conference agrees that memory is not encoded in base sequence in RNA.

The problem basically is what kind of biochemistry might conceivably relate electrical input to a permanently altered posture of a unit within our memory register. The unity of biochemical devices from the lowest to highest cells in creatures encourages us to raise the thought that the hardware of the neuron and of the brain is constructed and operated with the same logic so successfully used in the solution of other developmental and evolutionary life problems. Looked at in this light, our first thought will certainly be that electrical input to a neuron derepresses a particular gene whose function it is to be derepressed by this particular modality of effector. Derepression of such a previously repressed gene will result in increased RNA synthesis as is observed. The gene once derepressed may

stay derepressed for ever more, thus causing a permanent change in posture of the unit, the encoding of a unit of permanent memory.

Let us then be somewhat more specific about a model of the chemical changes involved in the storage of information in permanent memory. We think today of memory in terms of registers in which information is displayed in binary form. This mode of thinking is almost certainly over-simplified but it must do until some more sophisticated model comes along. Thus, we have initially an empty register, one that contains no information. As a result of learning, the register is reset, some information is displayed. The elements of the register are either individual neurons or more probably individual synaptic junctions between neurons. Resetting of an element of the register means that a change in the electrical properties of that element have taken place. In the case of the short and medium term memories, such change is reversible and in fact reversed by time as well as by electrical activity. In the case of permanent memory, the changes in electrical properties associated with the resetting of the unit of the register are permanent ones. Let us consider a neuron and a single synapse; a unit involved in encoding in permanent memory. A signal is transmitted through the synapse and causes the neuron to fire. Our problem is how to, as a result of such an event, permanently increase the probability that a signal from the same synapse will result in the firing of the neuron in future. We will assume that the DNA-dependent synthesis of RNA is somehow involved and we know, in fact, that the excitation-induced RNA synthesis possesses the earmarks of derepression of genetic activity, for example, the increase in amount of such synthesis. We already know from neurophysiology of one way in which electrical signals are transformed into chemistry. An electrical impulse travelling down an axon reaches the end of the fibre. There it finds a number of small bags of

transmitter substance; one or more bags are ruptured by the arriving impulse and acetylcholene, or some similar transmitter thus released, is free to diffuse across the synaptic junction and depolarize the membrane of the adjacent dendrite. The derepressing substance of memory cannot be acetylcholine since the experiments of Bennett and his colleagues at the University of California, Berkeley, have shown that alterations in acetylcholine and choline-esterase in cortical tissue as a result of learning are small indeed. My own candidate model is one which envisages that the dendrite also contains small bags - bags containing preformed material of small molecular nature. One candidate to be contained in such bags might be gamma amino butyric acid, a substance unique or nearly so to brain tissue. These bags are then to be broken by the act of transmission across the synapse. The substance thus released is, in the first place, perhaps a sensitiser of the dendrite for synaptic conduction and in the second place an effector substance for the derepression of genes to make the messenger RNA to make the enzymes to make more of itself. Since a single cortical neuron may have 100 or more synaptic inputs, it is possible that one hundred or more different effector substances and their relevant genes may operate in a single neuron, each specific to a particular synapse. But, on the other hand, let us not make our model over-specific because to do so would almost certainly render it incorrect. Let us summarize its principal features by saying that the learning-induced chemical alterations in the neuron which result in permanent memory and which appears to be associated with the synthesis of new RNA may very well be the derepression of a previously repressed gene or genes for the ultimate manufacture of substances which render more sensitive, or less sensitive, to conduction a particular synapse or synapses. Our model possesses the virtue that it makes predictions which are subject to experimental test and in

fact to simple tests. It predicts, in the first place of course, that the act of storage in the permanent memory should be inhibitable by actinomycin-D. There has been as yet no thorough test of this matter. Our model predicts in the second place that the RNA made during the learning process should be a gene product and be therefore hybridisable with the genomal DNA. It is in contrast in this respect to the "memory molecule" view of cortical RNA which would envisage the learning-induced RNA as made by non-DNA-dependent RNA synthesis. On this view, the learning-induced RNA should, of course, be non-hybridisable with the genomal DNA. And finally, our model predicts that the learning-induced RNA, since it is made by transcription of genes previously repressed, should be different from the RNA made in the same cell in the absence of learning. This last prediction gets at the very heart of the question of whether storage in permanent memory is associated with derepression of genes. An extraordinarily desirable experiment along this line would be that of hybridisation competition experiments using pulse-labelled RNA from the cortices of sensorily deprived rats and of sensorily affluent rats. If learning and memory are associated with gene derepression, the pulse labelled RNA of sensorily affluent rats should contain new species distinguishable by hybridisation; different from the species present in the pulse labelled RNA of sensorily deprived rats. The outcome of this experiment will tell us with certainty and rigour whether or not derepression is at work in the memory process.

Finally, the present Conference has been extraordinarily fruitful. It has summarized and codified present information concerning the chemical alterations in the cortex involved in learning and memory; it has brought general agreement among the participants that changes in RNA and protein levels are involved in the encoding of information in permanent memory; and

it has suggested for the future lines of thought and particular and specific experiments which it may be hoped will be useful in future unravelling of the problem. A further Conference, if held in two years time, should be able to summarize vast progress in the field of the molecular biology of learning and memory.

SOME NOTES ON THE STRUCTURE OF MEMORY

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Introduction

The title of this conference is Macromolecules and Complex Behavior,
yet interestingly, though we have heard a lot about macromolecules, we
have not heard a word about complex behavior. No one has even mentioned
it. I think the most complicated task that was presented was a visual dis-
crimination task (black vs. white) performed by rats. This is not exactly
"complex" behavior. So we've had two interesting days on macromolecules
and I guess I am expected to wind up with something on complex behavior.
I am not, of course, going to talk about all kinds of complex behavior,
but will restrict myself to the memory mechanism. About four years ago
I wrote a paper on "The Search for the Engram" (In Progress in Neurology
and Psychiatry, New York: Grune & Stratton, 1961, 45-57), and noted that
in the decade since Lashley had made his negative comments a good deal of
knowledge had accumulated and that the door was now open to a "decade
of decision" in coming to grips with the problems.

The last two days have borne my prediction, despite the confusion
always produced by new data. I want to look at the memory process in two
ways this morning. One is to focus the content of memory and the other is
to study its structure. Most of my own work has been on the content of
memory, and I won't belabor the details of these experiments again here.
But I do want to point out that we do not perhaps expect a memory process
to be the same when we are dealing with auditory memories as when we are
dealing with visual memories; when we are dealing with memory necessary to
accomplish a sequence of events; when we are dealing with telephone numbers;

or when we are involved in the memory mechanism operative in the perform-
ance of sexual behavior or food ingestion. We have assumed throughout
this symposium that all of these things are the same and this assumption
is unwarranted. We must keep in mind that there are a great number of
studies and literature that point to different neural mechanisms for each
of these behaviors. But I won't discuss these mechanisms here because
they are only remotely related to the problems of macromolecules. Yet
these facts must always be at the back of our minds--always we must be
aware of the question: "memory for what?", not just memory per se.

The Structure of Memory

Nonetheless when we discuss the role of macromolecules in memory we do
make an assumption of some sort of homogeneity--some sort of basic alike-
ness in learning and remembering, irrespective of these differences in
content. And to some extent this assumption may indeed be valid. Let us
call the assumption the structure of memory. What does this structure
look like? This has been the question before us during the last few days.

1. Permanent Storage: It is undoubtedly true that an organism can
react differentially on the basis of a temporally remote experienced oc-
currence. I have had monkeys perform perfectly tasks learned several years
earlier with intervening experience only on other types of tasks. Skinner
has shown that pigeons retain a visual discrimination over a two year per-
iod without intervening practice. The human counterpart needs no recount-
ing. What then are the changes in the organism that allow this long term
change in performance?

    a. Inputs may either be isomorphically recorded or they may be
coded into programs which when properly activated may reconstruct "mem-
ories." Three observations support the recording of these hypotheses. One
is the occurrence of eidetic imagery; another is the phenomenon of hypnotic

regression. Evidence in the latter situation is poor and has been devastatingly reviewed (Martin Orme and Theodore Barber). A final one is the evokation of "memories" by electrical brain stimulation and I have already noted yesterday that this happens only in epileptic (i.e. scarred) cortex and is subject to influences of environmental set (George Mahl). Further, the accuracy of report to reported situation is in doubt. That leaves eidetic imagery: its occurrence in the adult is extremely rare and considering the interest such a phenomenon must arouse in psychologists, studies on eidetikers are practically nonexistent.

The evidence is overwhelmingly in favor of the suggestion that most of the memory process is like a machine. Bartlett (Remembering, Cambridge: Cambridge Univ. Press, 1932) amply documented this view that schemata rather than items of memory are stored in the head. There is a considerable difference between the two models. If storage were isomorphic to experience you should be able to locate and find correspondences between stored items and the world out there. In a schematic or programmed memory no such isomorphic relations would obtain. The difference is that between a dictionary and a typewriter, the difference between a trigonometry table in a handbook of physics and chemistry and a calculating machine. This difference is of vital concern to us here. Let's say I take a simple adding machine and I add the capability to multiply. In the process of making it a machine that can also multiply, I'm putting another memory unit in it. I have thus modified the machine which could previously only add, so that now it can multiply. If I look into the machine I will find a change and that change may be the addition of a set of registers. Yet I will never find any specific product by opening the machine. Products are always obtained by presenting the machine with certain inputs. Much confusion would be resolved if we adhered to the notion that remembering is

the opposite of dismembering. Even our language reflects that remembering is a putting together, a reconstruction. This means that a good deal of what we call the memory storage problem is a hoax. Most memory is stored in our libraries as inputs to such brain machines--in our jobs and homes as inputs to the human organism who remembers what he is triggered to remember. Our very word "remember" reflects this process of reconstructing from parts as by a machine--the opposite of "dismember." A caution, however; in no way do I want to imply that man is nothing but a machine. Man does more than just remember. And keep in mind also that I have been talking about permanent storage. I'm talking about one kind of memory. I'm not saying that you can't memorize telephone numbers for a couple of hours, or visualize temporarily an image just experienced.

b. As to mechanism. Once we dispose of the hoax that isomorphic coding and recording of inputs takes place we can also get rid of the numbers game that we have heard played here and elsewhere. Bits of information are irrelevancies--every book an author writes can be "stored" in his typewriter which possesses fewer than 50 symbols on its registers. You can argue that the brain must be more complicated than a typewriter and I agree-but the number of symbols or states that its registers can display is an experimental rather than a logical or psychological question. An alphabet of only 26 letters does a heroic job.....

I have repeated these by now almost truisms, because I find that in our discussions we do not hold this model dear. Over and over the argument turns around storage of particulars, as it did yesterday and the day before. These need not be 10^{10} units for storage, there need not be an RNA change specific to a Y maze but not to a T maze. Only if the model one holds is one that demands item storage--the storage of inputs in some isomorphic manner--is this kind of argument valid. And the evidence

is overwhelmingly against such bit-by-bit storage.

c. Despite these difficulties in ridding ourselves of conceptual shackles, progress _is_ being made by leaps and bounds. Hyden's work has been criticized here by psychologists and by biochemists; yet, the picture he began to draw for us is taking form. The RNA changes he reports may indeed be occurring--not as evidence of item storage on evanescent messenger molecules--but as evidence of derepression of genomes. Bonner's theory and Hyden's evidence are in accord. But what then of cannabalism and the injection of "knowledgable" RNA. This is the focus of discrepancy--here _experiment_ must attack. The evidence must be firmed; new directions taken to decipher the relationship between RNA and derepression. As of now, an increase in RNA signals that derepression has occurred--could it also be that RNA in some way can initiate derepression?

d. This elegant theory is not enough, however. Changes in neural registers in and of themselves are insufficient to account for memory unless a changed circuitry is accomplished as has been repeatedly mentioned. Could this change in circuitry be due to a change in the basic temporal code with which the nerve discharges--or to which it is sensitive--as demanded by Hyden's and Hebb's theories as well as by some of my own notions? The experimental evidence for any such a temporal code specificity is lacking though within the capabilities of our techniques and is urgently needed. When available, it will give us a look at the "machine" which can generate the specific memories demanded by the input situation.

e. Does this then dispose of the neural or synaptic growth or strengthening hypothesis? Not necessarily. As I pointed out on another occasion (The New Neurology: Memory, Novelty, Thought and Choice. In EEG and Behavior, New York, Basic Books, 1963, 149-173) the electrocon-

vulsive shock experiments have provided evidence that consolidation of the memory trace is at least a twofold process. Immediately or for five seconds--even up to an hour after--all traces of an experience can be wiped out. Perhaps the derepression mechanism is reversible during this period. After this the register changes take place but unless there are alterations in circuit design, retrieval -i.e. the activation of this particular matrix of registers of this particular machine--becomes awkward if not impossible, except in the most restricted of situational inputs. Thus the derepression--RNA register mechanism may well be supplemented through growth or some equivalent increase in connectivity. So much for permanent memory.

2. _Intermediate, Flexible, Memory Processes_. As I already indicated there are memory processes which do not acquire the permamence discussed above. Memorization of telephone numbers in a strange city, the use of experience in a related but novel situation, the schedules which guide us through our daily tasks and pleasures--the recrudescence of extinguished performances when the conditions of extinction are lifted--these are some of the temporary rearrangements to which the more permanent input register is subject. How are such rearrangements accomplished? What assures flexibility? As yet no biological mechanism has been proposed to account for flexibility. Nor will I attempt to propose one in detail today. But we do know that lesions of the limbic forebrain and the anterior frontal isocortex impair these types of memory processes. I have elsewhere (A Progress Report on the Neurological Process Disturbed by Frontal Lesions in Primates. In _The Frontal Granular Cortex and Behavior_, New York: McGraw Hill, 1964, 28-55) suggested that this deficit is due to a failure in the regulation of the time constant of a "hold" imposed by an input on a particular matrix of registers. This is assumed to be accomplished through an

operation similar to that which gives rise to a temporary dominant focus in the experiments of Ukhtomski, Rusinov and Morrell. Without regulation by such a hold mechanism, the organism fluctuates inordinately among matrices so that temporary combinations, i.e. registrations, cannot be achieved.

Implicit in this suggestion is the idea that our memory machinery is capable of hierarchical organization--that all small units and probably some larger combinations of machinery are permanent and undamageable, but that at least some of the larger units can be flexibly combined through programming operations, initiated either in the input or by the larger permanent units. Also implicit is the suggestion that a particular memory unit can serve in a variety of combinations and thus participate in the production of a variety of remembrances. Such equipotentiality is, however, limited as our experiments on memory content have demonstrated (Toward a Science of Neuro-Psychology (Method and Data). In Current Trends in Psychology and the Behavioral Sciences. Pittsburgh: Univ. of Pittsburgh Press, 1954, 115-142).

Also implicit in this view of the intermediate hierarchical arrangement of the memory mechanism, is that memory is all of a piece; and that we can enter the hierarchy at any point. For instance, I could have talked today to you about intermediate memory first, recognition memory second, and long term memory last. I have all of these things to say to you and it's only my output mechanism which can only do one thing at a time. This limitation on output is "the keystone in the construction of the individual" as Sherrington has so beautifully stated it. Serial ordering prior to output is therefore a part of this memory machinery. What possible role do macromolecules play in this process? Could it be, as we dared hope during this conference, that a more or less temporary sensitization of some neurons

or some particular patterns of synaptic connections occurs--a sensitization akin to those found elsewhere in the organism which produce immunities? Here, as we found out, we do not even know how to ask the proper experimental questions. But again a lead comes from the laboratory. I have had occasion to cause epileptic seizures in monkeys by implanting aluminum hydroxide cream in their cortex. A major convulsive episode will leave performance of a task unimpaired in these animals. Only 24 to 48 hours after such an episode has ceased does performance deteriorate--this in the absence of further seizures. Some process takes these many hours to build up sufficiently to challenge the otherwise dominant learned patterns of neural response. Could this observation be usefully employed to get at these "intermediate" memory processes?

3. Recognition Memory. Finally I must pose a problem which in many respects is very different from the two preceding ones. I have already alluded to the problem in the discussion--viz--look at a friend--recognize him--then look at his neighbor, also a friend--and immediately he too is recognized. In the auditory mode such transient, rapidly paced recognitions of phrases in music, of phonemic combinations of speech are the commonplace. Permanent matrices of registers and even their flexible combination cannot account for the immediacy and evanescence of memory in awareness. Here a still different mechanism must be in operation. What could it look like?

Let me begin by detailing a paradox. The paradox concerns habit and habituation. If we are repeatedly in the same situation, in invarient unvarying environment, two things happen. One is that if we have to perform a task in that environment, that task becomes fairly automatic, it becomes more efficient. We say the organism has learned to perform the task, he has formed habits. At the same time the subject habituates, by

which we mean that he no longer produces an orienting reaction. That is,
he no longer notices the events in his environment. His verbal reports
of introspection, his failure to move his head and eyes in the direction
of the stimulus, electrophysiological measures such as galvanic skin re-
sponse, plethysmography and EEG, all attest to the disappearance of or-
ienting with repetition of input in an unvarying situation. Habituation,
however, is not an indication of some loss of sensitivity on the part of
the nervous system. Sokolov has demonstrated that if he decreases the
intensity of a tone which has been repeatedly given to a subject, orienting
or alerting will recur. Further, if he again habituates the subject and
shortens the duration of the tone, orienting again will take place. This
time to the unexpected silence. These things led Sokolov to propose that
a neural model of the environment is produced in the nervous system. This
is an expectancy, a type of memory mechanism against which inputs are con-
stantly matched. The nervous system is continually tuned by inputs to
process further inputs.

Of course, the habitual performance of an organism is also due to
neural activity. The point is that in the case of tuning there is a dim-
inution of neural activity while in the case of performance, enhancement
seems to occur. So the question is what is the difference between these
two kinds of neural activity that appear to be inversely related to each
other.

My suggestion is that the graded potential changes of nerve tissue
and the nerve impulse make up the two kinds of processes involved. The
channeling of nerve impulses obviously is related to performance. Graded
neural events must therefore be involved in the orienting reaction and its
habituation. How?

A synapse doesn't work by itself. Nerve impulses arrive at many

synaptic-dendritic junctions simultaneously. In essence such arrivals generate fronts. Such wave fronts, once established, can interact, producing Morray or interference patterns. My suggestion is that the orienting reaction is a function of such interference patterns.

Subjectively, the orienting reaction is correlated with awareness, habituation with unawareness. What evidence have we to suggest that the graded electrical activities of the central nervous system are involved in awareness? Joe Kameia at the University of California Medical School in San Francisco, has shown, using instrumental conditioning techniques, that people can be aware of whether their brains are producing alpha rhythms or not. My suggestion, therefore, is an old-fashioned one--that we experience some events going on in the brain and not others. If this is so and the alpha rhythm experiments hold true, one must indeed be able to tell one brain pattern of graded potential changes from another. It thus is not too far fetched to assume that patterns of graded neural activity can be discriminated.

But if our observations are correct, nerve impulse patterns per se must be unavailable to awareness unless they generate graded potential patterns at synaptic and dendritic locations; but I would be hard put to prove this at this time. And here I think Augenstein's model of conformation change at the synapse comes in. Synaptic changes must be quickly reversible. Conformation changes would be exactly what are needed. Synapse could be preset to be sensitive to certain kinds of patterns through such a reversible conformation change. If the expectancy, the pre-setting, hasn't taken place the environment would go by in a whirl and would not be processed at all. The time constants of this kind of reversible change can be gotten at experimentally. If one looks at EEG records coming off an EEG machine for a number of hours during a day, and goes home to try to sleep,

what happens? The day's records go by in review; but they go by in reverse! Could protein conformations be pushed in one direction and then as they reverse "play the tape back" but in the opposite direction? And could this drift in memory reflect a drift of synaptically produced interference patterns?

Conclusion

So, in summary, we must, if we are to discuss both macromolecules <u>and</u> complex behavior be aware first of all of the content, i.e. the different functions which memory serves. In addition, the memory process itself must be analyzed. Its structure reveals at least three separable mechanisms: a long term store of programs capable of regenerating the items we remember; an intermediate flexible operation which, when suitably activated by inputs arranges the particulars of remembering; and a short term recognition process intimately tied to awareness. Macromolecules may indeed be involved in each of these processes as I have here somewhat wildly speculated. Perhaps some future conference can come to grips more precisely and with considerably more experimental backing with these specific relations. For "memory mechanisms" are no more monolithic in their structure than are "macromolecules."